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THE PRODUCTION OF 2,3 BUTYLENE GLYCOL BY AEROBACTER FERMENTATION

A. C. Blackwood

Department of Soils

University of Alberta

May, 1944

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Allister Clark Blackwood

Department of Soils

A THESIS

submitted to the University of Alberta

in partial fulfilment of the requirements for

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MASTER OF SCIENCE

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TABLE OF CONTENTS

Page

Introduction 1

Review of Literature 4

Outline of Investigation 13

Results 14

Study of *Aerobacter aerogenes* #199 14

1. Method of handling and storing cultures 14
2. Inoculum studies 17
3. Fermentation media 25
4. Temperature of fermentation 37
5. Time of fermentation 37
6. Control of pH 37
7. Nutrients 43
8. Aeration 46
9. Glycol-alcohol ratio 62
10. Yield Curves 64

Isolation and Studies of New Organisms 67

1. Isolation technique 67
2. Selection of suitable cultures 68
3. Glycol and alcohol yields 69

Discussion 72

TABLE OF CONTENTS (Continued)

Page

Summary 76

Acknowledgments 78

Literature Cited 79

Appendix 83

Note: In all tables where the designations 'A', 'B', or 'C' are used, they refer specifically to the same types of calculations as are defined in Table 1, page 20.

THE PRODUCTION OF 2,3 BUTYLENE GLYCOL BY *AEROBACTER* FERMENTATION

A.C. Blackwood

INTRODUCTION

Owing to the critical shortage of raw and processed rubber for both civilian and military requirements the development of a substantial synthetic rubber industry among the Allied Nations became essential early in 1942. The synthetic development was until then practically non-existent. The Baruch report to the Congress of the United States, considering both Canada and the United States, became the basis upon which the industry was developed on this continent.

Buna S type of rubber (37) is the most desirable and it is produced as a polymer of 30% styrene (by-product from petroleum industry) and 70% butadiene. Butadiene can be obtained from three sources:

1. From waste gases from the petroleum cracking industry (using very expensive high priority equipment with this cheap source of raw material.)

2. From acetylene from coke ovens (using a very long, expensive method of preparation involving a large source of electrical energy.)

3. From starch from corn or wheat (using comparatively cheap and lower priority equipment with this relatively expensive and

uncertain source of raw material.)

With the possibility of the exhaustion of the source from petroleum due to heavy competition in essential production of high octane gasoline, and the impracticability of the method using acetylene due to excessive costs, and also considering the necessity for industrial development of agricultural resources, the last source received much investigation by both industrial and technical men.

Two types of fermentation may be used to produce butadiene. The first type involves the fermentation of sugar medium by yeast to give ethyl alcohol followed by a conversion of the alcohol to butadiene. In the second type also two major steps are involved in the production of butadiene:

The first step involves the bacterial fermentation of agricultural products to give 2,3 butylene glycol. The second step is the conversion of the glycol* to butadiene. This latter fermentation process involves either starch or sugar media. The genus Aerobacillus appears to give the best results where starch medium is fermented while the genus Aerobacter is mainly used with sugar medium.

The work on this problem was organized in Canada and the United States and the Northern Regional Research Laboratory at Peoria became the centre of the development. Under the National Research Council at Ottawa the Soils' and Field Crops' Departments of the University of Alberta jointly undertook a laboratory study of the fermentation problem.

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* The term Glycol used here and elsewhere refers only to 2,3 butylene glycol.

The study was divided into three sections:

A. _Aerobacter_ fermentation (Soils Department)

B. _Aerobacillus_ fermentation (Field Crops Department)

C. Analysis of fermentation liquors (Biochemical section of the Field Crops Department)

Our problem was mainly concerned with obtaining standarized conditions of fermentation by which in repeated tests the results showed the greatest yield in the shortest time. Other observations made were considered to be incidental to the main aim of the project.

From time to time 'progress reports' were sent to Ottawa from all three laboratories. In March 1943 A. L. Shewfelt reviewed in his M.Sc. thesis (36) the results then obtained in all sections of the project.

This thesis covers only fermentation of the _Aerobacter_ type on sugar or starch-hydrolyzed media. A. W. Jackson is reporting on _Aerobacillus_ fermentation and W. G. Corns on the analytical aspects of the project, in their respective theses, so that the whole project will be completely considered at this time. A.L. Shewfelt (36) reported on the results from all three sections for the period May, 1942 to February, 1943. Only the investigations carried on since January, 1943 are here reported.

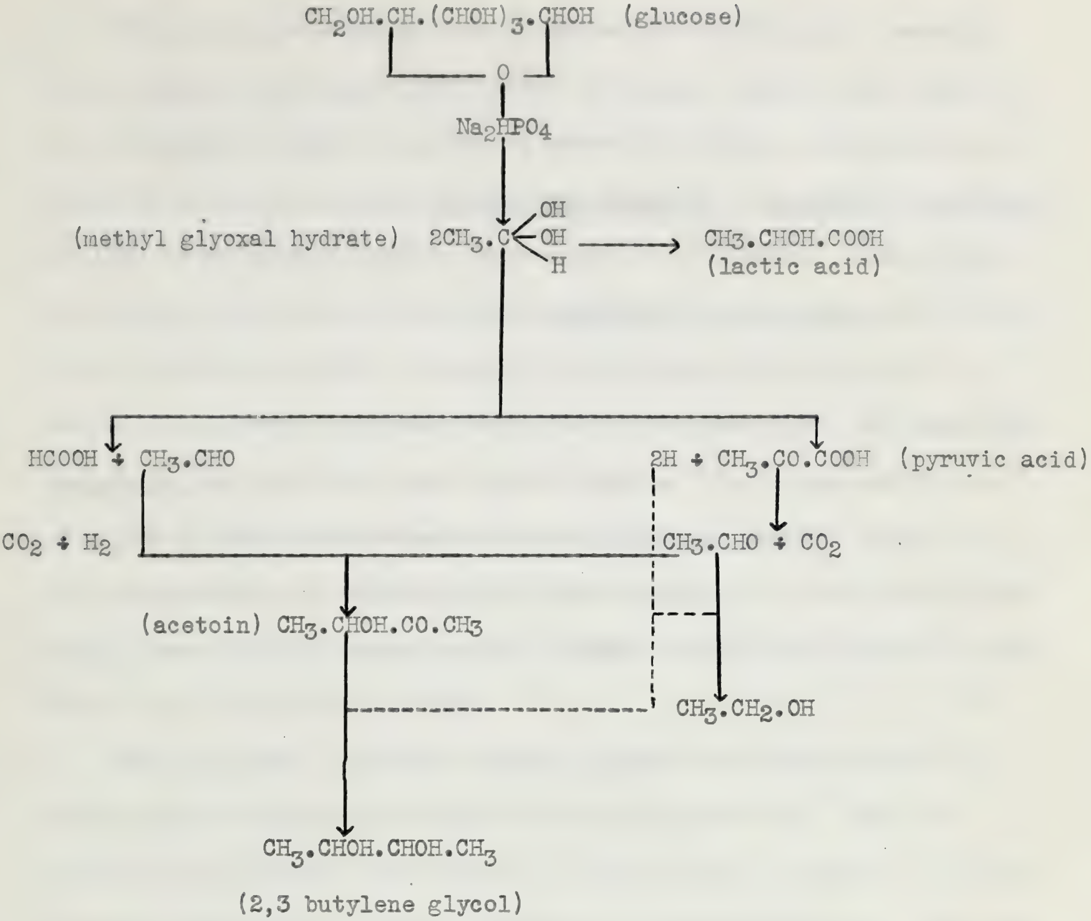
REVIEW OF LITERATURE

The Aerobacter genus is universally distributed in nature, being very abundant in soil and plant products. It is also found in the intestines of man and animals. This genus has received much investigation because it is a member of the 'coliform' group. The sanitary bacteriologist is very much concerned with its identification especially in relation to the presence of sewage in the water supply. The enormous amount of literature related to this genus that may be found is almost entirely concerned with the problems of sanitation. Many easy complete tests for identification have been developed and widely accepted.

In Bergey's Manual of Determinative Bacteriology (2) Aerobacter aerogenes, the type species, is listed as a short rod, 0.5 -0.8 by 1.0-2.0 microns, occurring singly, motile or non-motile, non spore-forming, frequently capsulated and gram negative. On agar medium the colony is thick, white, raised, moist, smooth, entire, more convex than Escherichia coli and often mucoid. On the agar slant growth is abundant, thick, white, moist, glistening and spreading. Acetylmethylcarbinol is produced giving a positive Voges-Proskauer test. The methyl red test is negative. Acid and gas are formed in dextrose, levulose, galactose, arabinose, lactose, maltose, raffinose, cellobiose, starch, dextrin, salicin, esculin, glycerol, mannitol, sorbitol and inositol. Sucrose, mannitol, inulin, pectin, dulcitol and adonitol may or may not be fermented. It produces two or more times as much CO_2 as H_2. It is a facultative aerobe and has an optimum temperature of 30°C.

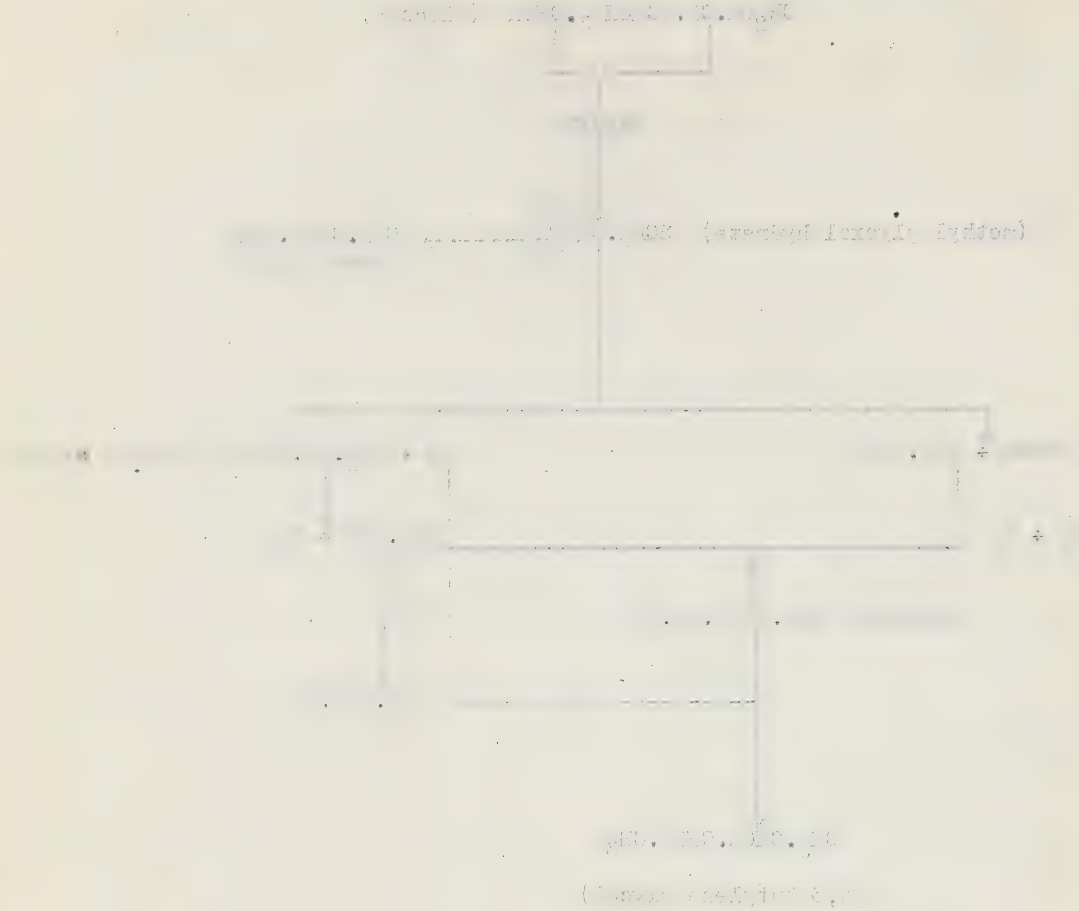
It is only in recent years that much attention has been given to the type of fermentation that this organism produces. Kluyver at Delft University, Holland patented in 1924 a method by which it is possible to obtain 2,3 butylene glycol commercially by fermentation from carbohydrate media. He and his co-workers investigated the type of fermentation produced and drew up a scheme that he thought best represented the metabolic breakdown from dextrose in the fermentation (22). This scheme is given below. It is realized that this representation is by no means completely accepted by all investigators. Since 1929 Fulmer, Werkman and their associates at Iowa State College have been concerned with the metabolic breakdown of carbohydrates in this and other fermentation processes. Reynolds and Werkman (29) suggest by a series of equations the probable breakdown route as determined by a number of experiments with Aerobacter indologenes. It is not within the scope of this review to attempt to evaluate the suggested schemes. Kluyver's scheme is given because he demonstrates in a relatively simple manner the general type of intermediates formed and their role in the metabolism of the substrate. Note the suggestion in this diagram that glucose does not form methyl glyoxal hydrate directly but is 'phosphorylated' in a similar manner to the phosphorylation steps found in the metabolism of all carbohydrates by bacteria and yeast and in muscle. Anderson (1) discusses the theories of Kluyver, Neuberg and Meyerhof in this regard as related to carbohydrate metabolism by yeast.

Harden and Walpole (15) state that the products formed in the Aerobacter fermentation are formic, lactic, acetic and succinic acids, also H_2, CO_2, ethyl alcohol, acetylmethylcarbinol (acetoin) and 2,3 butylene glycol. Reynolds and Werkmann (29) confirm this.



FERMENTATION OF GLUCOSE BY *AEROBACTER AEROGENES*

(as suggested by A.J.Kluyver)



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Some early investigators were interested in obtaining a satisfactory fermentation to give good yields of glycol. Verhave (39) patented in England in 1928, a process by which 2,3 butylene glycol was produced by fermentation using Clostridium polymyxa or Aerobacter aerogenes. In 1929 he patented a further improvement in the method. Fulmer et al (14) studied the fermentation using Aerobacter pectinovorum and attempted to get an optimum medium. Kendall (20) continued this study and he listed the effect of various factors on the fermentation. He found four Aerobacter cultures that gave similar results. In optimum conditions only 50% of the sucrose consumed was converted to glycol. Kihara (21) developed methods of isolation and identification of glycol and carbinol. Using three different organisms and a simple medium the fermentation was found to be completed in 14 days.

No large scale intensive research program had been developed to investigate the commercial aspects of this fermentation. When this study became essential the N.R.R.L. of the U.S.D.A. at Peoria, Illinois, gathered together the existing knowledge of the fermentation. With the close cooperation of interested research and commercial laboratories N.R.R.L. became the centre to which progress reports were periodically sent and likewise from which reports were distributed.

N.R.R.L. was able to obtain yields of glycol approaching the maximum amount obtainable in both laboratory and pilot plant tests, using their isolate Aerobacter aerogenes #199. An agar medium and standard methods of handling the culture (26) were suggested. Heyden Chemical Corporation (16) in studying this isolate noted that under heavy aeration the cells were short and thick but using light aeration the cells were longer, larger and irregular. Several laboratories (4, 31, 38)

isolated Aerobacter cultures which they tested, but no isolate was reported that gave significantly better results than #199.

Much of the reported research reviewed the factors which affect the fermentation. It was hoped by careful control of standardized fermentation conditions at all times to obtain maximum yields.

N.R.R.L. (26) suggested using 5 - 10% of a 24 hour inoculum in their fermentation medium. Heyden Chemical Corporation (16), using their own suggested inoculum, shaken for 18 hours at 32°C., found that optimum results were obtained where a 5% inoculum was added. The University of Wisconsin (38) found that size or age of inoculum had little effect and they used 5% of a 12 hour N.R.R.L. inoculum. Repeatedly transferring the culture gave no increase in yields. The Seagram Laboratories (33) and the Schenley Research Institute (30) both suggest that 5% of an 18 - 20 hour 'rich' inoculum is successful. Schenley Research Institute (31) suggests making four transfers in inoculum medium from the slant; the last transfer is to be to an aerated fermentation mash with added nutrients. This is to 'acclimatize' the culture.

Many fermentation media have been suggested but the simple medium of N.R.R.L. (20) has been most widely used. It was found essential to have small amounts of $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 in all dextrose media. N.R.R.L. (26) found that salts were not essential in malt-hydrolyzed corn mash. Schenley Research Institute (30) suggested that if salts were added to the inoculum then they were not needed in the fermentation medium. Urea was reported by all investigators to be the best source of nitrogen. The amount added varied from 0.2 - 0.3%. Birkinshaw et al (3) found that $NaNO_3$, microcosmic salts, NH_4NO_3, $(NH_4)_2SO_4$ are utilized, but that $Ca(NO_3)_2$, and

$NaNO_3$ are not. Fulmer (13) suggests using NH_4Cl as the source of nitrogen in the production of glycol from sucrose medium with Aerobacter faeni. All investigators suggest the separate sterilization of $CaCO_3$ either dry or in a slurry. N.R.R.L. (26) and University of Wisconsin (38) both found that corn steeping liquor added to the medium increased the fermentation rate; however N.R.R.L. reported that the yield of glycol was decreased up to 10% by this liquor. Lucidol Corporation (23) reported that the addition of vitamins had no effect. University of Wisconsin (38) suggested the beneficial use of unheated malt sprouts but Seagram's (33, 34) found no such results.

University of Wisconsin (38) reports that Cu and Fe are detrimental to the fermentation at a concentration of 2.00 mg. per litre. Seagram's (33) investigating the effect of metallic ions, found that Cu was most toxic. Concentrations of 0.1 - 0.5 mg. per litre of Cu suppressed growth. They found that Ni, Fe, Zn, Cr, and Sn, inhibited the culture but that Mg, Ag, and Hg stimulated the organism at low concentration. Heyden Chemical Corporation (18) reported that adding sugar concentration up to 20% plus phosphate and urea gave good yields but Commercial Solvents (6) reported poor returns where sugar concentrate was added.

Malt-hydrolyzed corn mash was tested with varying success by many laboratories (6, 18, 26, 30). It was found that the saccharified mash must be sterilized as the contamination introduced by the malt affected the fermentation greatly. Merck and Co. (25) tried using Ryzopus delemar as the saccharifying agent with little success. N.R.R.L. (26) using formaldehyde -treated malt and moldy bran got poor results. Some laboratories (16, 18, 25, 30) used acid hydrolysis and obtained a desirable medium.

A fermentation temperature of 30°C. was suggested in all cases. The time for normal fermentation giving good results varied from 40 to 72 hours approximately. The suggested pH range is from 5.0 to 6.5.

Aeration is apparently the most important variable factor in the fermentation. Its role is by no means explained but its effect is well known and easily demonstrated. In estimating the effect of aeration on the fermentation the following observations must be considered:

In the inoculum sample the numbers of bacteria are more rapidly increased by shaking or aerating the sample (38). In small samples stirring with a mechanical mixer was reported by some laboratories (16, 31, 38) to give good results. Adding a hydrogen acceptor (methylene blue) does not satisfactorily take the place of aeration (32, 36). The National Research Council at Ottawa (4) reported that nitrogen may be used effectively as the aerating agent but this was not verified elsewhere. The same amount of air may be more effective if broken up into smaller bubbles (38). Less air is required for the same volume of ferment where the cross sectional area is reduced and the depth thus increased(38). Oxygen is not needed to balance any equations demonstrating the breakdown of sugar to glycol (22, 29).

Thus it is felt that aeration exerts a physical rather than a chemical action in the fermentation.

Many aeration rates have been suggested as optimum. The condition of the culture, the type of fermenter, the volume of the medium and the desired time of fermentation all affect the amount of air required. N.R.R.L. (26) suggests a rate of 20 cc. per litre per minute in a rotary drum fermenter, and a rate of 100 - 700 cc. per litre per minute in a vat fermenter. The aeration is stopped when the glucose level reaches

1.0%. Schenley Research Institute (30, 31) reports that 510 cc. of air per litre per minute gave the best results and that when the sugar level had dropped to 3% aeration was to be discontinued. Where shaking is used 90 cycles per minute is reported to give good results (16, 38). By varying the amount of liquid in the flask the amount of aeration can be varied. Various aerators were suggested, such as gas diffuser stones (16), cotton bags (18, 38), carbon aerators and pipes with small holes (18). The University of Wisconsin (38) suggest a formula for calculating the amount of air necessary for a proper fermentation.

Columbia Brewing Company (5) investigated the feasibility of adapting a brewery type plant to this fermentation but secured very low yields with a 99 hour fermentation. (Their results show that the mash was too acid for normal growth.) Heyden Chemical Corporation (17) by using first a 1000 gallon stainless steel kettle and later (after 18 hours) wooden tungs, secured good yields. Jos. E. Seagram and Sons developed a detailed scheme for large scale fermentation and first tested the scheme in the pilot plant.

Some of the yields obtained by various investigators are given below.

N.R.R.L. (26) found that by using Aerobacter aerogenes #199 they obtained yields on the average in which 80 to 88% of the sugar consumed was converted to glycol. 0.5 - 1.0% alcohol was formed. An average of 12.75 lbs. of glycol per bushel of corn was obtained (theoretical yield is 18.9 lbs. per bushel of corn). Using Aerobacillus polymyxa with corn mash as a substrate N.R.R.L. (27) obtained 10.5 lbs. of glycol and 6.4 lbs. of alcohol per bushel of corn. Schenley Research Institute Inc. (31) obtained as their best yield, in Aerobacter fermentation, 4.62% glycol in the final liquor which is 16.2 lbs. of glycol per bushel of

corn.

P.J. Kolachov of Jos. E. Seagram's and Sons (35) states that in Aerobacter fermentation it is possible to obtain 8.2 lbs. of butadiene per bushel of grain (assuming 16 lbs. of glycol per bushel, 95% recovery of the glycol, and a 90% conversion to butadiene). At that time he stated that it was possible to get 6.4 lbs. of butadiene (assuming 15 lbs. of glycol per bushel, 95% recovery of the glycol and 75% conversion to butadiene). Using ethyl alcohol as the intermediate product only 5.5 lbs of 85% pure butadiene is obtained per bushel. Butyl alcohol used as an intermediate can give not over 2.1 lbs. of low purity butadiene.

A comparison of these results shows the value that the Aerobacter type of fermentation will have in any comprehensive plan concerning the production of butadiene, by fermentation.

OUTLINE OF INVESTIGATION

The definite aim of this investigation is the study of the *Aerobacter* type of fermentation with a view to securing maximum yields of 2,3 butylene glycol in the shortest possible time. The research is divided into two major divisions:

1. A greater part of the work dealt with the organism *Aerobacter aerogenes* #199, the selection obtained through Ottawa from Northern Regional Research Laboratory at Peoria, Illinois. This fermentation was carried out with aeration in most cases. A variety of conditions was studied and an effort was made to obtain optimum yields consistently.

2. The second division was concerned with isolations of *Aerobacter* organisms from local sources in soil, vegetables etc. Most of this study involved no aeration.

As selection #199 was known to give a quick fermentation with good yields the main portion of the research has been devoted to a study of this organism. Conditions suggested as optimum elsewhere have been tested and the results compared. Numerous other aspects were investigated and all results are reported below.

The analysis of the fermentation liquors for sugar, 2,3 butylene glycol, acetylmethyl carbinol and ethyl alcohol was carried on by members of the Biochemical section of the Field Crops Department. A complete report of the methods used and an evaluation of the experimental results obtained are reported by W. G. Corns in his thesis (7). Estimation of pH was done in this laboratory using a Beckmann glass electrode pH meter.

RESULTS

Studies of Fermentation using *Aerobacter aerogenes* #199

1. Method of handling and storing the culture.

The culture has been stored on agar medium suggested by N.R.R.L. (26) and made as follows:

Difco tryptone	5 gm.
Difco Yeast Extract	5 gm.
Agar	15 gm.
Glucose	1 gm.
Distilled water to make	1000 cc.

At first the culture was stored in the refrigerator (2°C.) to prevent too rapid drying, but later it was stored in the incubator at 30°C. and transferred every 10 days or two weeks.

An experiment to determine the degenerative effect, if any, due to improper storage conditions or too frequent transferring is reported by A. L. Shewfelt (36). No apparent degeneration in the culture occurred even after storing in the refrigerator for 8 months. A later experiment (see under Inoculum Studies, Table 2) showed that a slant stored in the refrigerator for 6 months gave lower yields.

It was soon realized that variations in the activity and fermentation ability of the culture from time to time led to serious discrepancies in results, and also that in commercial practice this variation would be much more serious. To offset this effect a modified lyophile method of drying the culture was tried as suggested by Dr. G. A. Ledingham, Division

of Applied Biology, National Research Council, Ottawa. This is a relatively cheap, easy laboratory method of accomplishing this type of storage on a small scale. The method suggested is a modification and combination of the so called 'lyophile' and 'cryochem' methods of Flosdorff and Mudd (10, 11).

It involves freezing the culture, suspended in blood serum in a suitable ampoule with a cotton plug, quickly at -70°C. in a mixture of dry ice and methyl cellosolve (acetone may be substituted). Following this, the culture (a large number may be lyophilized at one time) is placed in a vacuum dessicator containing a good dessicant (eg. Drierite) in an insulated container. It is kept in the frozen state for at least 4 or 5 hours and by the use of a Cenco hyvac pump the pressure is maintained below 0.5 mm. mercury. After leaving frozen at this pressure overnight the ampoules will be completely dried. They should be light colored and nearly the original volume. Remove from the dessicator and fasten the ampoules to a manifold. Again evacuate with the pump to a pressure of 0.5 mm. or less. Seal off the tubes at the constrictions with a torch. Label. Test for vacuum using a spark coil vacuum tester. Store at room temperature.

When needed scratch the ampoule with a file and break open inside a clean cotton pad moistened with alcohol. Drop the pellet into an inoculum or other nutrient solution. The pellet is extremely soluble. Note: See the accompanying photograph of apparatus and ampoules (Plate 1).

Insufficient time has elapsed since beginning this study to estimate the value of this method of storing cultures. It is not known how long the culture of Aerobacter will be preserved by this method but it is hoped that in addition to retaining the cultures in their original [illegible] pplied Biology, [illegible] molecularly [illegible], easy laboratory method of [illegible] on a small scale. [illegible] of the so called '[illegible] [illegible]ff and [illegible] ('[illegible], II).

[illegible] freezing the culture, [illegible] dry ice and ethyl cellosolve (acetone may be substituted). [illegible] this, the culture (a [illegible]) [illegible] placed in a vacuum desiccator containing [illegible] in an insulated container. It is kept at [illegible] 4 or 5 hours and by the use of a Hyvac pump the pressure [illegible] tained below 0.5 mm. mercury. [illegible] overnight the ampoules will be completely dried. They [illegible] collected and [illegible] the original volume. [illegible] the ampoules to [illegible] pressure of [illegible] or [illegible] with a [illegible] [illegible] there [illegible].

Other uses [illegible] s [illegible] and resistant [illegible] bacteria or other [illegible]. [illegible] from the [illegible].

[illegible] this has [illegible] the value of this method of drying [illegible] for long [illegible] [illegible]

It is hoped that in addition to [illegible]

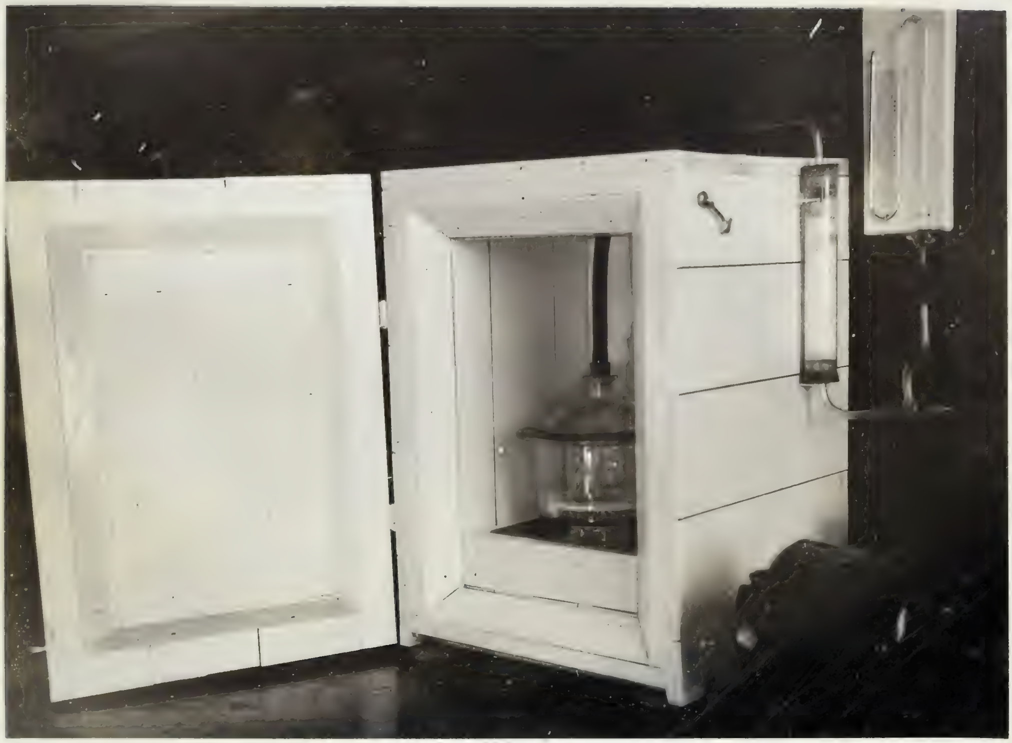


PLATE 1.

(Photograph of Apparatus.)

condition of activity, a further advantage will result from a reduction in the amount of routine labour necessary to preserve our isolates.

2. Inoculum Studies

The inoculum medium generally used was that suggested by N.R.R.L. (36) containing:

Glucose	100 gm.
Conc. Corn Steeping Liquor	5 gm.
$CaCO_3$	5 gm.
Distilled water to make	1000 cc.

Two other suggested inoculums have been tried:

Seagram's

Beef Extract	3 gm.
Peptone	10 gm.
Yeast Extract	10 gm.
Malt Sprouts	75 gm.
Dextrose	150 gm.
$CaCO_3$	5 gm.
Distilled water to make	1000 cc.

Schenley's

Dextrose	150 gm.
Beef Extract	10 gm.
Tryptone	5 gm.
Yeast Extract	10 gm.
Urea	3 gm.
Malt Sprouts	5 gm.

$CaCO_3$............................... 5 gm.

Distilled water to make.......... 1000 cc.

These three inoculums were compared and as far as could be determined by fermentation results no decided advantage was obtained by the use of any particular inoculum. The N.R.R.L. inoculum was mostly used because it is easier to prepare and shows less tendency to cause foaming. However all three have given good results.

Growth cruves as determined by the plate count method were obtained for the following inoculum treatments:

A. N.R.R.L. inoculum run without shaking at 30°C.
B. " " " with " " 30°C.
C. Seagram's " " without " " 30°C.
D. " " " with " " 30°C.

The shaking rate varied from 90 to 120 strokes per minute. Counts were made up at 4, 8, 12, 16 and 25 hours. Standard methods of sampling, pouring plates and counting were followed. Na caseinate agar suggested by Fred and Waksman (12) was used and the plates were examined on the third day. Two duplicate tests were run pouring in one case three and the other five plates at each dilution. The results are shown on the accompanying graph (Plate 2).

Shaking gave much higher numbers than non-shaking and Seagram's inoculum slightly lower counts than N.R.R.L. inoculum. However as shown in Table 1 no consistently better yields were obtained in the fermentation by shaking the inoculum than non-shaking. In these and other experiments it was felt that other factors (eg. rate of aeration, pH etc.) that are not so easily controlled exerted a more definite

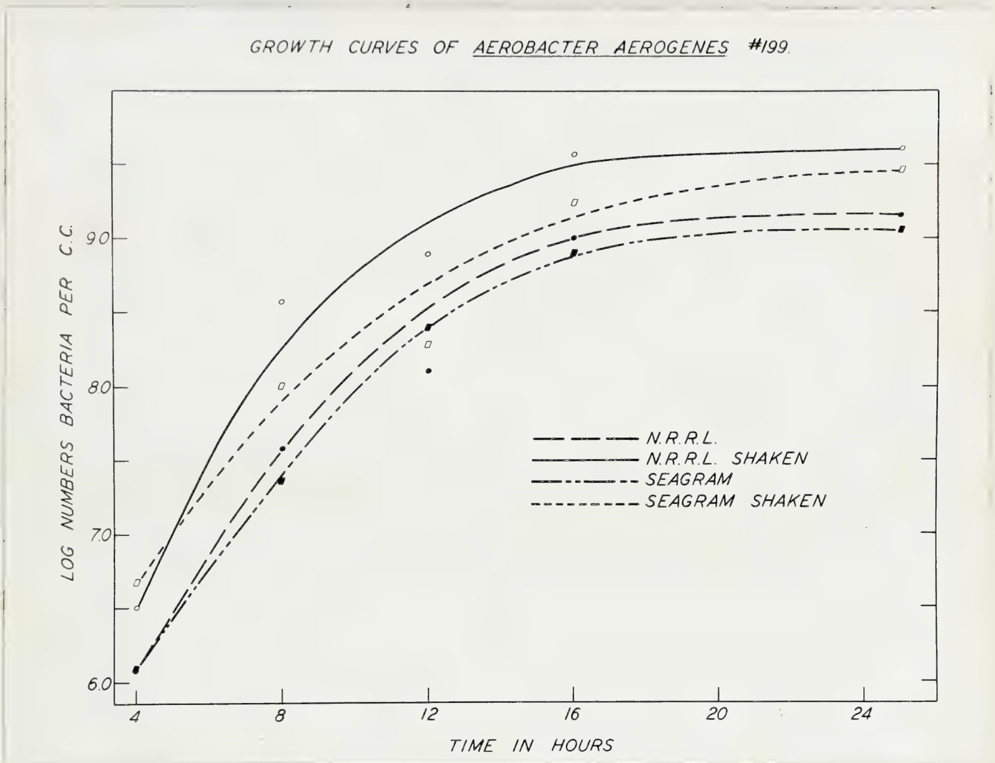

GROWTH CURVES OF AEROBACTER AEROGENES #199.
LOG NUMBERS BACTERIA PER C.C.
9.0
8.0
7.0
6.0
4
8
12
16
20
24
TIME IN HOURS
N.R.R.L.
N.R.R.L. SHAKEN
SEAGRAM
SEAGRAM SHAKEN

PLATE 2

TABLE 1

EFFECT ON 2,3 BUTYLENE GLYCOL YIELD OF SHAKING OR AERATING THE INOCULUM

Inoculum	Treatment	Medium	No. of Samples	% Sugar Final	% Sugar Consumed	Glycol	'A'*
A. Time of fermentation = 40 hours							
N.R.R.L.	No Aeration	dextrose	12	2.87	8.53	2.77	65
"	Aeration	"	6	1.22	10.18	3.66	72
B. Time of Fermentation = 46 hours							
N.R.R.L.	No Aeration	dextrose	6	2.81	8.34	3.45	83
"	Aeration	"	6	1.08	10.07	3.99	79

* 'A' in all tables gives the amount of glycol formed compared with the theoretical amount that could be formed from the sugar consumed, expressed as a percent.

'B' in all tables gives the amount of glycol plus acetylmethylcarbinol formed compared with the theoretical amount that could be formed from the sugar consumed, expressed as a percent.

'C' in all tables, gives the amount of glycol plus carbinol plus alcohol formed compared to the theoretical amount that could be formed from the sugar consumed, expressed as a percent.

influence on the yields.

Frequent transferring (daily) using inoculum medium for two months increased the activity of the culture and the rate of gas production. The inoculum became thick at the end of 24 hours. However it did not increase the rate of fermentation beyond a certain point that can be reached by keeping the culture in a relatively fresh condition by occasional transfers. This thick inoculum in fact caused somewhat slower fermentation rates and bad foaming occurred. Some of the results are shown in Table 2.

By the isolation of colonies from the culture of frequently transferred inoculum or agar plates it was possible by randomized sampling to obtain certain strains which had the characteristic of producing a thick or syrupy inoculum. When these isolates were slanted the resultant growth was very slimy. After discarding these isolates somewhat better fermentation results were obtained. Table 3 shows the effect of this selection of strains using dextrose medium and moderate aeration. In this case eight colonies, after being described, were selected and incubated. Two further sets of plates were poured to test the purity of the culture with respect to colony form. The strains were then slanted and fermentation tests run. Only #3 strain showed the typical syrupy characteristic and gave poor results, probably due to difficulties in aeration.

This characteristic syrupy growth has been eliminated once or twice since by a further selection of strains after plating the culture.

Another experiment testing the method of handling the culture was carried out on three media: dextrose, malt-hydrolyzed Marquis granular flour, and malt-hydrolyzed Garnet granular flour. As the method of

[illegible]

[illegible]

[illegible] it did not increase the rate of fermentation [illegible]

point that can be reached by cooling the culture [illegible]

[illegible]

[illegible]

As the position of colonies from the surface of [illegible]

inocula or agar [illegible] it was possible by individual [illegible]

certain strains [illegible]

[illegible]

[illegible]

[illegible] were obtained. Table 3 gives the [illegible]

strains [illegible] and [illegible] the mutation. In this [illegible]

[illegible]

[illegible]

TABLE 2

EFFECT OF THE METHOD OF HANDLING THE CULTURE ON THE FERMENTATION OF DEXTROSE MEDIUM

Source of Inoculum	Medium	% Sugar		pH	%	%				
		Final	Consumed		Glycol	Carbinol	% Etoh	'A'	'B'	'C'
A. Time of fermentation = 45 hours										
Slant	Sugar	0	11.0	-	3.87	-	-	70	-	-
"	"	.69	10.3	-	2.82	-	-	54	-	-
"	"	0	11.0	6.5	2.75	.35	.40	50	56	64
Frequently transferred culture	"	.93	10.1	-	2.69	-	-	53	-	-
	"	.78	10.2	-	2.67	.12	-	53	55	-
	"	.72	10.1	5.3	2.42	.15	.14	48	51	54
B. Time of fermentation = 65 hours Rate of aeration = 2000 cc. per litre per min. (Results given are an average of 5 ferments in each case.)										
Slant	Sugar	.14	9.7	5.4 to 6.2	3.42	.12	.55	71	73	84
Frequently transferred	"	1.03	8.8	5.0 to 5.5	3.16	0	.52	72	72	84

TABLE 3

EFFECT OF SELECTED STRAINS OF #199 ON THE YIELD OF GLYCOL FROM DEXTROSE MEDIUM

Strain Number	Number of runs	% Glycol
1	4	2.05
2	4	1.90
3	8	1.86
4	9	2.21
5	9	2.68
6	4	2.09
7	4	2.21
8	9	2.17

analysis of malt sugars then in use was later proved wrong, the original percent sugar in the malts is unknown in these tests. The experiment was run in triplicate but showed wide variations among the replicate samples. The results are given in Table 4 as an average of the replicates.

Methods of handling the culture and inoculum.

(a) Transferred culture in inoculum medium every day for 30 days before using.

(b) As (a) but aerated inoculum last 24 hours.

(c) Transferred from fresh slant to inoculum, transferred for 3 days using inoculum medium, before using.

TABLE 4

EFFECT OF THE METHOD OF HANDLING THE CULTURE ON THE FERMENTATION OF DEXTROSE AND MALT-HYDROLYZED MEDIA

(Only the final percent 2,3 butylene glycol reported.)

Medium	Culture					
	a	b	c	d	e	f
Dextrose	3.70	3.31	3.20	4.66	3.24	3.71
Malt Marquis	3.16	2.88	2.65	2.40	0.51	2.96
Malt Garnet	3.49	3.74	2.39	2.26	0.12	2.98

(d) As (c) but aerated last 24 hours.

(e) Culture from old slant stored in the refrigerator for 6 months.

(f) Culture from fresh slant to inoculum transferred to inoculum once and used.

Only the percent glycol found in the final fermentation solution is reported in the table.

The results vary so widely that it is impossible to draw definite conclusions. Storing the culture in the refrigerator (note (e)) without transferring is obviously deleterious. Frequent transferring appears to give no better results than using a fresh slant. The same result may be noted in Table 2B. Other variables seem to exert more of an effect than that of handling the inoculum in any definite manner.

A. L. Shewfelt (36) reported that a 20% inoculum gave better yields than a 6% inoculum or 10% inoculum; however later experiments have

shown that as low as 5% inoculum under optimum fermentation conditions gave satisfactory yields.

The estimation of numbers of bacteria in an inoculum solution (see Plate 2) showed that optimum numbers were reached at 16 hours, but the use of a 16 hour inoculum as compared to a 24 hour inoculum did not give significant differences either in rate of fermentation or yield of 2,3 butylene glycol.

For convenience usually 5 - 10% of a 24 hour inoculum is added to the fermentation medium and this practice appears to give satisfactory results.

3. Fermentation Media

Much of the fermentation study has involved the use of a sugar medium although it is definitely realized that the commercial possibilities may involve the use of starch from grain. One of the main objections to the Aerobacter type of fermentation is that starch is not fermented by the culture or at least only very slowly or in small amounts. Thus a starch medium must be hydrolyzed before becoming available.

1. Glucose Media

More consistent results can be obtained by using dextrose medium than by using a hydrolyzed starch medium. The pH and foaming tendencies are more easily controlled. A more uniform medium is obtained for comparison over a long period of time. The effect of nutrient supply can be more easily measured. Media suggested by N.R.R.L. (26), Seagram's Laboratories (32) and O'Meara (28) have been tested. The constituents of these three are listed below.

N.R.R.L.

Glucose	100 - 200 gm.
$MgSO_4 \cdot 7H_2O$	0.25 gm.
KH_2PO_4	1.80 gm.
Urea	2.00 gm.
$CaCO_3$	5.00 gm.
Distilled water to make	1000 cc.

Seagram's

Meat Extract	3 gm.
Tryptone	5 gm.
Difco Yeast Extract	10 gm.
Urea	2 gm.
Malt Sprouts	10 gm.
$CaCO_3$	5 gm.
Distilled water to make	1000 cc.

O'Meara

NaCl	5.0 gm.
$MgSO_4 \cdot 7H_2O$	0.2 gm.
$NH_4H_2PO_4$	1.0 gm.
K_2HPO_4	1.0 gm.
Na citrate	2.77 gm.
Glucose	5.0 gm.
KNO_3	10.00 gm.
Distilled water to make	1000 cc.

In Table 5 some of the results from experiments using these media are given. Note the exceptionally low yields using the O'Meara medium. The cause of the lowering of the 2,3 butylene glycol yield when this

TABLE 5

Effect of N.R.R.L.'s, O'Meara's and Seagram's Media on the Fermentation

Medium	# Samples	% Sugar Final	% Sugar Consumed	pH	% Glycol	'A'
A. Time of fermentation = 66 hours						
N.R.R.L.	5	0.0	8.4	-	2.94	70
O'Meara	5	0.0	8.4	-	0.49	12
B. Time of fermentation = 118 hours						
N.R.R.L.	3	2.1	8.9	5.6	3.83	86
Seagram	3	3.8	10.6	5.2	3.99	75
C. Time of fermentation = 70 hours						
N.R.R.L.	3	0.72	9.3	5.4	4.01	87
Seagram	3	0.54	8.9	5.7	3.72	84

medium is used is not easily explained. The effect is undoubtedly associated with the salts supplied. The products produced instead of glycol are not known.

Seagram's medium appeared to be as favorable for growth as that of N.R.R.L. although the comparable yields are somewhat lower. Seagram's medium has another effect not shown in this table and that is its tendency to foam under heavy aeration. This effect is induced probably by the addition of protein and protein decomposition products. As N.R.R.L. medium is simple in its requirements and gives satisfactory results most of our experiments have been concerned with this medium.

The addition of malt sprouts was suggested by a University of Wisconsin report (38) in which it was stated that the addition of 3% unheated malt sprouts definitely stimulated the fermentation. Heating the malt sprouts destroyed this effect suggesting that the effect was produced by a thermo-labile factor. Seagram's Laboratories (33) however found no such stimulating effect from the use of sterile or non-sterile malt sprouts. To test this a sample of malt sprouts was secured by the courtesy of the Canada Malting Company in Calgary. Some of the results obtained are given in Table 6.

These results show that sterilized malt sprouts have apparently no effect on the yield and any differences noted are probably due to other variable factors. Unsterilized malt sprouts however have a very noticeable effect on the fermentation. Apparently a very active alcohol producing contaminant is introduced which does not inhibit the growth of the *Aerobacter* culture. This is definitely shown where no inoculum was added.

[illegible]

[illegible] single [illegible] requirement [illegible]

[illegible] definitely stimulated the [illegible]

[illegible] found to [illegible] effect [illegible]

[illegible] malt [illegible]. In [illegible]

[illegible] by the [illegible] of the [illegible]

[illegible] The results obtained are given in table.

[illegible]

TABLE 6

EFFECT OF MALT SPROUTS ON THE FERMENTATION OF DEXTROSE MEDIUM

		#	% Sugar		%	%				
Malt Sprouts	Inoculum	Samples	Final	Consumed	Glycol	Carbinol	% Etoh	'A'	'B'	'C'
A. Time of fermentation = 63 hours										
0	+	2	0.54	9.5	2.98	0.59	0.47	63	75	85
3% sterile	+	2	0.52	9.5	3.01	0.74	0.57	63	79	91
B. Time of fermentation = 68 hours										
0	+	4	0.36	9.5	2.90	0.00	-	61	61	-
3% sterile	+	4	0.24	9.7	2.35	0.00	0.56	49	49	60
C. Time of fermentation = 69 hours										
0	+	2	0.35	6.5	2.03	0.00	0.40	63	63	75
3% sterile	+	2	0.00	10.0	2.17	0.00	0.70	43	43	58
3% non-sterile	+	2	0.63	9.4	3.02	0.00	0.45	64	64	74
3% non-sterile	-	2	2.33	7.7	0.94	0.00	2.05	24	24	78
10% non-sterile	-	2	0.00	10.0	0.91	0.00	1.99	18	18	58

TABLE 7

EFFECT OF AUTOCLAVING SOME DEXTROSE SOLUTIONS ON pH

Sample	No.	pH of original solution	pH after autoclaving at 15 lbs. for 15 minutes.
Batch 1	1	5.8	4.5
" 1	2	5.9	4.5
" 2	3	5.9	4.3
" 2	4	5.9	4.3

Thus it is felt that malt sprouts exert no beneficial effect on the fermentation and have not proved useful under the above conditions.

Some difficulties with sugar solution becoming acid after autoclaving have been noted and the medium is difficult to neutralize because of the lack of buffering capacity. If the sugar is pure dextrose this phenomenon is hard to explain. This effect is noted only with some batches of dextrose. For example, two different samples of sugar were autoclaved in duplicate, using distilled water, and the results are shown in Table 7. This phenomenon introduces some difficulties in buffering the medium and at the same time keeping the medium sterile.

2. Starch-hydrolyzed Media

Wheat has been used as the source of starch in all cases. Various methods of preparation have been used. Ground wheat, granular flour, fine flour and relatively pure starch have all been prepared

and hydrolyzed. Due to the variability of our results and the relatively small differences in starch content of local wheat as reported by McCalla and Corns (24) no large experiments were carried out on the grades, varieties and sources of wheat in our fermentations. Garnet, Marquis and Red Bobs wheat grown on Edmonton black soil were used.

Ground wheat was prepared by grinding a cleaned sample in a Wiley mill with a fine screen.

In the laboratory mill G. Tosh of the Field Crops department milled granular flour. He had some difficulty in getting a sufficient amount of bran out of the flour and still retaining the granular form. It was found that this type of product has some very definite advantages in handling ability over a finer grind of flour or pure starch. Because of the granular grind it was comparatively easy to obtain a smooth mix with water and this was not true with flour or pure starch.

Flour was obtained by the regular milling process carried out in the Field Crops Department.

Starch was produced by a very slow method of washing stiff flour dough in a fine stream of water and discarding the gluten residue. After allowing the starch to settle, the supernatant fluid was drawn off and the starch spread out to dry. When dry the scale is scraped off and ground in a hand mill. By this method a comparatively pure sample of starch was obtained. The method was slow, tedious and very messy, thus limiting the amount of work carried out on this medium. However some pure starch was obtained from the National Research Council at Ottawa considerably augmenting our supply.

There are three recognized methods of hydrolyzing starch to sugar; by the use of acid, malt and moldy bran. Only the first two methods

have been tried here, as yet.

(a) Acid hydrolyzed media

Using acid hydrolysis the methods found to give the best results are described by A.L. Shewfelt (36). These suggested methods did not produce a consistently uniform product and led to variable fermentation results. However it is realized that in good commercial practice using high pressure equipment (40) starch may be easily and conveniently acid hydrolyzed, giving a very even and reliable product. This method was eliminated in our work in favour of using malt.

(b) Malt hydrolyzed media

Through the cooperation of the Canada Malting Company in Calgary, samples of both brewer's (100° Lintner approximately) and distiller's (200° Lintner) malt have been obtained. Distiller's malt gives better liquefaction and in some cases at least better saccharification than brewer's malt, though this is hard to measure accurately. In the preparation of the malt W. Brown in the Biochemistry section did some research and the results show that freshly ground malt mixed with sufficient water and thoroughly stirred in a mechanical mixer for 20 minutes gives as high a Lintner value in the liquid extract as freshly ground malt mixed with water and allowed to stand at room temperature for two hours. Storing the extract in the refrigerator for 24 hours gives little loss of enzyme activity (approximately 6%) whereas keeping the extract at room temperature for 24 hours greatly reduces the B-amylase activity (approximately 18%). These extracts prepared in the mixer showed greater losses than those prepared by standing at room temperature, perhaps due to an inhibitory action produced by the metal container.

The medium is made up in 2-litre batches; 375 gm. of ground wheat,

granular flour or starch is added with good stirring, using a small electric mixer, to about 1500 cc. of cold distilled water. The temperature is raised in a water bath to 70°C. and gelatinization takes place. 1% malt sprouts mixed with water is added with good stirring until liquefaction of the starch has taken place (5 to 15 minutes). Then the mash is transferred to a 2 litre florence flask and autoclaved for one hour at 22 lbs. pressure. Special precautions must be taken to prevent boiling over during the autoclaving. When cooled to 60°C., 5% malt steep is added, complete mixing is ensured and the mash is left in a 55°C. oven overnight. If desirable, the mash is then filtered using a flat filter with two layers of cheese cloth. The clear filtrate or the original mash is then autoclaved and used for medium when desired. This method gives a fairly uniform product that is easily reproducible in the laboratory. Using granular flour good results have been obtained. With care in autoclaving there is rarely a need of a preliminary buffering of the medium. Reducing sugars in this medium approach the maximum amount obtainable.

The results obtained using the various media are reported elsewhere under separate headings. It may be stated that the most important factor in regard to starch-hydrolyzed media is pH, and a very close control of this factor is necessary. It was found by microscopic examination of glucosazone crystals that both maltose and glucose were present at the beginning and end of a fermentation involving malt-hydrolyzed medium, although the percent concentration of each was not known. Apparently the organism can utilize maltose as readily as glucose.

[illegible] mixed with [illegible] 1000 cc. of cold [illegible] water. The [illegible] in a water bath to 70°C. [illegible] minutes [illegible] liquefaction of the starch has taken place (15 to [illegible] minutes). Then the mash is transferred to a 2 liter [illegible] and [illegible] for one hour at 20 lbs. pressure. [illegible] cooled to 50°C., [illegible] malt [illegible] is added, [illegible] and the mash is left in a 50°C. oven overnight. [illegible] mash is then filtered using a [illegible] filter with two layers of [illegible]. The clear filtrate [illegible] and [illegible] was observed. [illegible] have been obtained, with [illegible] regard in this [illegible].

The [illegible] of [illegible] using this [illegible] which [illegible] conditions. It may be [illegible] that [illegible] factor in regard to [illegible], [illegible] of this factor is [illegible]. [illegible] of rhizopus [illegible], although the [illegible]. [illegible] the organism [illegible].

The initial percent sugar is very important in determining the percent 2,3 butylene glycol in the final liquor. Various levels of initial percent sugar in both dextrose and starch-hydrolyzed media have given a variety of results. Some difficulties, in the preparation of a starch mash containing more than 15% by weight, were noted due to the gelatinizing properties of the medium preventing good mixing. The percent conversion of the starch to sugar was low in these cases. Experiments involving the addition of dextrose concentrate at intervals during the fermentation did not give proportionate returns. In most fermentations the initial sugar concentration of the medium was about 10%. Some of the results are reported in Table 8.

It was suggested in a report from Lucidol Corporation (23) that 2,3 butylene glycol has a depressing effect on the activity of the culture. This inhibitory effect of 2,3 butylene glycol on the fermentation was studied by the addition of small amounts of glycol (0 - 3%) to the medium at the time of inoculation. Dextrose medium was used and urea, salts and $CaCO_3$ added. Medium aeration was supplied. The results were so variable that, in all, five experiments with duplicates in each were run. The results are given in Table 9 as an average of the 5 sets of duplicates. Plate 3 shows this effect graphically. In any one test the yields do not give a smooth curve and great variability is noted in considering single results, however, averaging all the results gives the effect shown. It is definitely felt that the inhibitory action of 2,3 butylene glycol is one of the main limiting factors in the use of a higher initial percent sugar.

The most suitable medium for this fermentation will undoubtedly be limited by the commercial aspects of the enterprise.

TABLE 8

EFFECT OF INITIAL SUGAR CONCENTRATION ON THE FERMENTATION

#	% Sugar			Time	# Sam-	%	%	%			
	Initial	Final	Consumed	(hours)	ples	Glycol	Carbinol	Alcohol	'A'	'B'	'C'
A.	3.0	0.00	3.0	69	10	0.95	-	-	63	-	-
B.	7.6	1.06	5.5	70	6	2.57	0.11	-	93	97	-
C.	8.6	6.50	7.9	64	10	3.31	-	-	86	-	-
D.	8.9	0.64	8.3	45	2	2.58	0.50	-	62	74	-
	8.9	1.14	7.8	69	2	2.47	0.50	-	63	76	-
	*13.7	1.43	12.3	69	2	3.23	0.35	0.62	53	58	72
E.	9.8	0.59	9.2	65	10	3.29	0.06	0.54	72	73	85
F.	11.0	0.55	10.4	45	6	2.87	0.20	0.23	55	59	64

* Initial percent sugar was 8.9 but added sugar concentrate at 24 hours to give 13.7% total sugar.

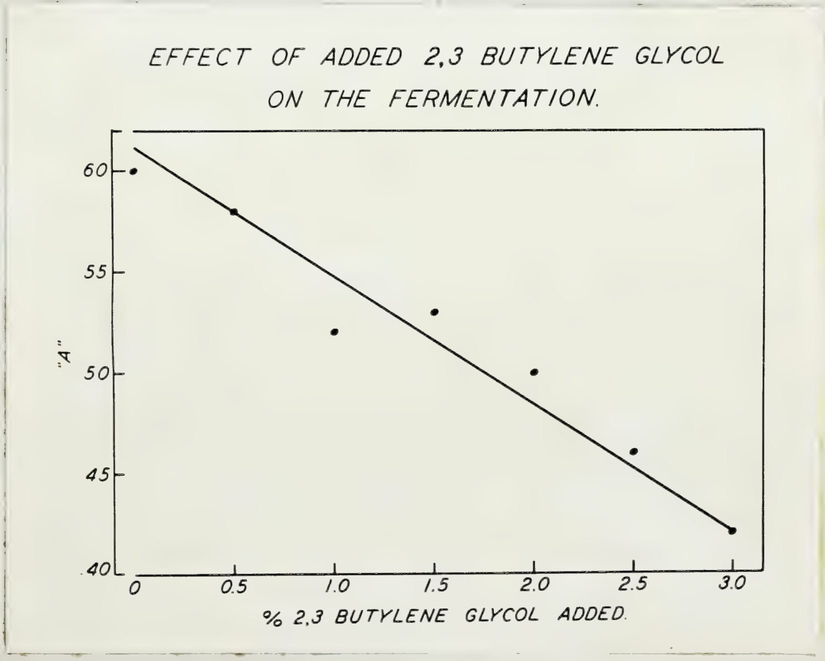
TABLE 9

EFFECT OF ADDED 2,3 BUTYLENE GLYCOL ON FERMENTATION OF DEXTROSE MEDIUM

Time of fermentation = 69 hours.

% glycol added	# Samples	% Sugar Final	% Sugar Consumed	% Glycol	% Glycol less amt. added	'A'
0.0	10	1.57	9.41	2.79	2.79	60
0.5	10	0.22	10.74	3.64	3.14	58
1.0	10	1.81	9.15	3.41	2.41	52
1.5	10	1.71	9.25	4.07	2.57	53
2.0	10	1.45	9.50	4.62	2.62	50
2.5	10	1.80	9.16	4.53	2.03	46
3.0	10	1.13	9.83	5.09	2.09	42

PLATE 3



4. Temperature of Fermentation

A number of tests were run at various temperatures in order to determine the effect on yield and rate of fermentation. In the range 30°C. to 36°C. no definite differences were found and temperature did not appear to be a limiting factor. Results from two different experiments will be found in Table 10. These results are at variance with those reported by A. L. Shewfelt (36).

As it is more convenient to keep the bath at 30°C. this temperature has been used in all cases, except where otherwise noted.

5. Time of Fermentation

While it is extremely important that the fermentation proceed to completion as rapidly as possible, the factors of yields must also be considered. The rate of fermentation is controlled by the condition of the organism and, more important, by the rate of aeration. Table 11 shows results obtained under somewhat similar conditions where the fermentation was completed in various lengths of time although the per-cent sugar was not reduced to zero in every case. It appears that 46 hours is too short a period for the fermentation and that at least 72 hours is necessary to secure reasonable yields. Too heavy aeration is detrimental to the yields

6. Control of pH

It is very necessary that the pH be carefully controlled in the fermentation. The range of pH at which the fermentation will be successful is from 5.0 to 6.5, with the optimum from 5.2 to 6.0. How-

TABLE 10

EFFECT OF VARYING THE TEMPERATURE ON THE FERMENTATION OF DEXTROSE AND MALT - HYDROLYZED MEDIA

Medium	Temperature (°C)	# Samples	% Sugar		pH	% Glycol	% Carbinol	'A'	'B'
			Final	Consumed					
A. Time of fermentation = 46 to 68 hours									
Sugar	32	3	1.09	7.8	5.6 - 7.6	2.22	-	65	-
"	35	3	1.51	7.4	5.8 - 7.0	2.45	-	66	-
Malt Mash	32	3	2.37	-	4.7 - 5.3	2.70	-	-	-
"	35	3	4.18	-	4.4 - 5.6	2.54	-	-	-
B. Time of fermentation = 46 hours									
Sugar	30	3	0.07	8.4	6.3 - 7.2	2.64	0.28	63	68
"	32	3	0.26	8.2	6.0 - 6.5	3.04	0.25	74	80
"	34	3	0.37	8.1	6.0 - 6.7	2.95	0.32	73	78
"	36	3	0.06	8.4	5.9 - 7.8	2.47	0.22	59	66
Malt)	30	3	0.09	-	4.9 - 5.1	2.95	0.00	-	-
Marquis(	32	3	3.51	-	5.0 - 5.7	3.04	0.00	-	-
"	34	3	2.35	-	5.0 - 5.1	3.78	0.09	-	-
"	36	3	2.66	-	4.9	2.00	0.00	-	-
Malt)	30	3	0.28	-	5.1 - 5.3	3.39	0.13	-	-
Garnet(	32	3	1.00	-	5.1 - 5.2	3.05	0.03	-	-
"	34	3	0.70	-	5.0 - 5.2	2.93	0.07	-	-
"	36	3	0.59	-	5.2 - 5.3	3.26	0.12	-	-

*

TABLE 11

EFFECT OF TIME OF FERMENTATION ON THE YIELDS OF GLYCOL, CARBINOL AND ALCOHOL FROM DEXTROSE MEDIUM

Date	Time of fermentation (hours)	Media	Aeration	# Samples	% Sugar Final	% Sugar Consumed	pH	% Glycol	% Carbinol	% Etoh	'A'	'B'	'C'
A. Experiment using same culture													
	118	Sugar	Low	3	2.1	8.7	5.6	3.77	.12	.40	87	89	99
	94	"	"	3	2.7	8.3	5.4	4.03	.15	.40	97	101	110
	70	"	"	3	4.7	6.0	-	2.94	-	.32	98	-	105
	70	"	Heavy then low	3	0.72	9.3	-	3.83	.28	.61	82	88	102
	43	"	Heavy	3	1.38	8.6	-	2.84	.30	.44	59	73	87
B. Experiments run at different dates													
June 30	40	Sugar	Heavy	18	2.30	9.1	-	3.11	-	-	68	-	-
July 8	46	"	"	6	1.26	7.9	-	2.67	-	-	68	-	-
" 9	46	"	Varied	15	1.43	8.3	-	2.64	-	-	64	-	-
" 22	63	"	"	9	2.61	7.4	-	3.09	-	-	84	-	-
Aug. 12	65	"	Heavy	10	0.59	9.2	-	3.29	.06	.54	72	73	85
" 16	68	"	Moderate	10	0.48	9.3	-	3.66	.51	.49	79	89	98

ever, under conditions of heavy aeration, even if the pH falls below 5.0 the fermentation will proceed until all the sugar is consumed, but the yields are very low. Under good fermentation conditions Aerobacter aerogenes #199 will ferment dextrose with no addition of $CaCO_3$ as a buffer. During the fermentation the pH falls from 6.0 to about 5.2 - 5.5 but sometimes does go lower. However the addition of small amounts of $CaCO_3$ is sufficient when needed to prevent the lowering of the pH in most cases below 5.0, using dextrose media. This is apparently affected by the condition of the organism which induces a variation in the amount of acid produced from time to time. Table 12 shows the effect of this variation on the yield.

Experiments run on June 22 and June 26, although the same amount of buffer was added to each sample, produced the variations in pH shown. Note that the higher comparative yields favor the lower pH in this case. From the results of an experiment on August 9 reported in Table 12, the samples were divided into two sections depending on whether the final pH was above or below 5.5. Considering the results, it is clear that although slightly higher yields were obtained at the higher pH there is no significant difference and thus it is assumed that a pH below 5.5 is not detrimental to the fermentative ability of the organism. However from the results of an experiment run on September 28, which was divided into two sections depending on the final pH, it is felt that the lower yield and high final sugar obtained below pH 5.0 are due to the inhibition of the culture by the acid reaction. These experiments were selected to show the effects mentioned and are representative of the influence on the culture of differences in pH.

The acid-hydrolyzed medium is very hard to control. The pH changes

TABLE 12

EFFECT OF CULTURE, BUFFER AND MEDIUM ON THE pH AND GLYCOL YIELDS

Date	# Samples	gms./100 cc.		% Sugar		pH	% Glycol	'A'
		$CaCO_3$	Na_2CO_3	Final	Consumed			
1. Dextrose Medium								
June 22	6	.8	0	.32	8.2	6.0 - 6.7	3.00	73
June 26	6	.8	0	.95	7.5	5.5 - 5.7	3.50	93
Aug. 9	6	.8	0	.77	9.0	5.0 - 5.4	3.19	71
	4	.8	0	.31	9.5	5.5 - 6.2	3.43	72
Sep. 28	3	.8	0	6.00	2.6	4.9 - 5.0	1.32	107
	2	.8	0	1.28	7.3	5.1 - 5.2	2.36	65
2. Acid-hydrolyzed Medium								
Mar. 2	4	0.8	.1	0.80	4.5	4.1 - 4.6	1.61	71
Mar. 12	1	0.8	.1	3.50	4.2	4.4	0.48	23
	1	0.8	.0	2.70	5.0	4.4	0.53	21
	1	1.6	.0	1.30	6.4	4.5	1.64	51
	1	0.0	.1	2.30	5.4	4.3	1.54	57
Mar. 17	2	0.8	.0	2.50	6.6	4.2 - 4.4	0.99	30
	2	0.8	.8	0.90	8.3	5.3 - 5.5	2.30	56
3. Malt-hydrolyzed Medium								
Sep. 15	1	0.8	.0	3.82	-	4.7	2.37	-
	1	0.8	.1	3.76	-	5.0	2.21	-
	1	0.8	.8	2.48	-	5.4	2.81	-
	1	0.0	.1	4.24	-	4.6	2.36	-
Sep. 28	2	0.8	.1	6.80	6.0	4.5 - 4.6	0.43	14
	2	0.8	.8	4.50	8.3	5.0 - 5.9	2.30	56
Oct. 13	1	0.8	.1	5.52	-	4.8	1.09	-
	1	0.8	.1	3.85	-	5.2	3.13	-
	1	0.8	.8	2.61	-	5.3	3.39	-
	1	0.8	.8	3.09	-	4.4	1.35	-

very rapidly during the fermentation and even if the amount of buffer added is high the medium often becomes acid. The amount of buffer needed varies greatly between experiments and a variety of results are found as shown in Table 12. Considering the results obtained in the four experiments listed it is apparent that a buffer consisting of 0.8% of $CaCO_3$ and 0.8% of Na_2CO_3 gave the best returns. The experiment carried out on March 2 showed low final sugar concentration, low pH and fairly good yield. The suggestion is that as the initial percent sugar was comparatively low in this test most of the sugar was utilized and 2,3 butylene glycol produced before the reaction became too acid. Thus the satisfactory yield was obtained. It is apparent, from the experiment on March 12 that no amount of $CaCO_3$ alone is sufficient to successfully buffer this medium.

Malt-hydrolyzed media is much easier to buffer. With care in autoclaving there is no need of a preliminary addition of alkali to obtain a satisfactory reaction. However even with this medium adequately buffered the pH has a tendency to vary between tests. Some of these variations are noted in Table 12. The buffer consisting of 0.8% $CaCO_3$ and 0.8% of Na_2CO_3 gave the best results. In some cases 0.8% $CaCO_3$ plus 0.1% Na_2CO_3 was sufficient to hold the pH at a high enough level to obtain satisfactory yields. $CaCO_3$ or Na_2CO_3 alone did not give sufficient buffering capacity to the medium. The results obtained on October 13 are an example of the unpredictable variations that may be found even in one experiment. It is this effect that introduces most of the difficulty in handling this malt-hydrolyzed medium in the laboratory and limits its usefulness in extensive tests.

7. Nutrients

1. Salts

Salts ($MgSO_4.7H_2O$ and KH_2PO_4) are very essential in the medium up to a certain level and must be added to the fermentation medium at the time of inoculation. Table 13A shows the effect of the presence or absence of salts on sugar and malted media and also the effect of adding salts to the inoculum as suggested by Schenley Laboratories (30). The beneficial effect of salts is not nearly as noticeable where malted solutions are used although some increase in yield is noted where the salts are added. In sugar medium the fermentation will proceed only to a limited extent in the absence of salts. In this experiment urea and $CaCO_3$ were added as suggested by N.R.R.L. (26).

In a previous extensive experiment the concentration of $MgSO_4.7H_2O$ was varied in steps of 0.0, 0.01, 0.02, 0.03 0.04 and 0.05% and at the same time the amount of KH_2PO_4 added was varied in steps of 0.0, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30%. These tests were run with aeration on acid-hydrolyzed starch, but the results were so variable that, no conclusion could be drawn from the experiment. The most apparent difficulty was in the control of the pH of the medium.

To counteract the variable effect of aeration an experiment was carried out with no aeration and the amounts of salts added to the fermentation medium were varied. The results are given in Table 13B. The experiment was run in triplicate and one sample analyzed at 3 days and the other two at 5 days. $CaCO_3$ and urea were added as suggested by N.R.R.L. (26). These results show that, at least, where no aeration is supplied, the amount of $MgSO_4.7H_2O$ and KH_2PO_4 necessary to carry on a normal fermentation approaches a very low level. This amount is

TABLE 13

EFFECT OF SALTS ON THE FERMENTATION OF DEXTROSE AND MALT-HYDROLYZED MEDIA BOTH WITH AND WITHOUT AERATION

A. With Aeration

Time of fermentation = 46 hours

Medium	Salts in Inoculum	Salts in Medium	# Samples	% Sugar Final	% Sugar Consumed	% Glycol	'A'
Dextrose	-	-	5	6.25	2.7	0.43	33
"	-	+	6	1.09	7.7	2.35	61
"	+	-	3	4.60	4.6	0.51	22
"	+	+	2	0.66	8.5	2.59	61
Granular	-	-	6	1.50	-	3.16	-
Flour	-	+	5	0.44	-	3.88	-
Malted	+	-	6	1.24	-	2.61	-
"	+	+	5	1.29	-	2.95	-

B. Without Aeration

% $MgSO_4 \cdot 7H_2O$	% KH_2PO_4	% Sugar Initial	% Sugar Final 3 days	% Sugar Final 5 days	3 days % Glycol	3 days 'A'	5 days % Glycol	5 days 'A'
0.01	0.05	8.2	0.5	0.3	2.51	64	2.67	67
0.01	0.10	8.2	1.0	0.3	2.20	61	2.74	69
0.05	0.05	8.2	0.5	0.3	2.44	63	2.93	73
0.05	0.10	8.2	0.1	0.3	2.67	65	2.67	67
0.05	0.10	5.6	1.0	0.1	1.33	58	1.67	60
0.05	0.50	5.6	0.1	0.1	1.80	64	1.68	60
0.30	0.10	5.6	0.1	0.1	1.74	62	1.73	62
0.30	0.50	5.6	0.1	0.1	1.82	65	1.75	63

somewhat below the level suggested in the N.R.R.L. medium. Certainly it will be unnecessary to supply the fermentation medium with salts in excess of the amounts suggested.

2. Nitrogen Source

Urea has been used as the source of nitrogen in nearly all the fermentations. It has proved entirely adequate and apparently is readily available to the organism at all times. In accordance with suggestions of N.R.R.L. the urea is added at the time of inoculation.

Other sources of nitrogen have been investigated briefly. NH_4Cl, $(NH_4)_2SO_4$ and to a lesser extent $(NH_4)_2CO_3$ have given successful fermentations. Growth is active and the sugar is rapidly utilized. However when using these salts the problem of buffering the medium to give a reasonable reaction becomes more difficult. The use of these sources of nitrogen presents no advantage in this fermentation and urea has proved to be entirely satisfactory from the laboratory viewpoint.

The beneficial use of non-autoclaved urea has been suggested by some laboratories (30). No significant gains or losses have been found in our experiments as a result of using either autoclaved or non-autoclaved urea. Urea is either dissolved in water and autoclaved for 15 minutes at 15 lbs. pressure or else dissolved in sterile distilled water and used without subsequent sterilization. Table 14A gives a comparative set of results using N.R.R.L. dextrose medium and adding urea at the time of inoculation. No real differences between treatments were found. The effect of autoclaving urea was also studied on malt-hydrolyzed solution with similar results, as reported in Table 14B.

The effect of various amounts of urea on a non-aerated fermentation

was determined. Varied amounts of urea were added to N.R.R.L. dextrose medium at the time of inoculation. The urea was dissolved in distilled water and autoclaved. The results are given in Table 15. The test was done in triplicate, one sample was analyzed on the third day and the other two on the fifth day and the results averaged. Although all the yields are low the higher amounts of urea give the better returns.

To test this observation an experiment was carried out, using aeration, under similar conditions. The results are given in Table 16. These results are reported as an average of triplicates. N.R.R.L. dextrose medium was used and salts and $CaCO_3$ supplied. The samples were analyzed at 40 and 49 hours. The effect on the pH of the fermentation medium of the various amounts of urea was very marked and the agreement between duplicate samples was exceptionally close. A very noticeable trend in sugar utilization and glycol yield is apparent at 40 hours. The higher amount of urea added definitely favors a faster fermentation rate. At 49 hours this effect is less marked. At the lower rates of application of urea the medium was acid enough to inhibit further fermentation. These lower samples were continued to 68 hours without any appreciable sugar utilization. It is realized that the range of urea used was very great and that 0.2% suggested by N.R.R.L. (26) is probably sufficient in most tests to give a normal fermentation.

However the observation that an additional amount of urea added will give a faster rate of fermentation with less danger of encountering an acid medium may be very important.

8. Aeration

Aeration is apparently the most important variable factor in the fermentation. In our experiments nearly all the variation among

TABLE 14

EFFECT OF AUTOCLAVING UREA ON THE FERMENTATION OF DEXTROSE AND MALT-HYDROLYZED MEDIA

Urea	# Samples	% Sugar Final	% Sugar Consumed	% Glycol	'A'
A. N.R.R.L. Dextrose Medium					
Autoclaved	9	2.59	8.8	3.04	69
Non "	9	2.05	9.3	3.18	68
B. Malt-hydrolyzed Marquis Granular Flour					
Autoclaved	6	3.35	-	2.67	-
Non "	6	2.86	-	2.69	-

replicates in the same test can be attributed to ineffective control of of aeration. A great number of experiments have been run using a variety of techniques and modifications of apparatus but, to date, no method has given consistent results. Two major aims were considered in all experiments regarding the effect of aeration. First it was hoped to obtain a simple, accurate method of controlling the amount of air supplied to each fermentation tube. Secondly it was desirable to obtain a greater understanding of the chemical or physical mechanism by which the aeration exerted its control on the fermentation. It was felt that the effect was a physical phenomenon and most of our tests were run with this consideration in view.

No system of control yet tried has given good agreement among replicates. In the laboratory where small samples are used a slight variation in the amount of air supplied has a very noticeable effect.

TABLE 15

EFFECT OF VARIOUS AMOUNTS OF UREA ON NON-AERATED DEXTROSE MEDIUM

%	% Sugar			3 days		5 days	
Urea	Initial	Final		% Glycol	'A'	% Glycol	'A'
		3 days	5 days				
0.05	8.2	1.0	1.0	1.09	30	1.45	40
0.10	8.2	1.0	1.0	1.58	44	1.65	46
0.40	8.2	0.5	0.5	1.87	48	2.36	62
0.50	8.2	1.0	0.5	1.62	45	2.49	67
1.00	8.2	1.0	0.5	1.76	47	1.97	52

TABLE 16

EFFECT OF VARIOUS AMOUNTS OF UREA ON AERATED DEXTROSE MEDIUM

% Urea	# Samples	% Sugar Final	% Sugar Consumed	pH	% Glycol	'A'
A. Samples analyzed at 40 hours.						
0.05	3	4.83	4.8	4.8 - 4.9	2.02	88
0.10	3	3.37	6.2	5.0 - 5.0	2.55	82
0.50	3	1.12	8.5	5.7 - 5.7	3.08	61
1.00	3	0.85	8.7	6.1 - 6.1	3.06	59
B. Samples analyzed at 49 hours.						
0.05	3	3.30	6.6	-	2.42	73
0.10	3	2.43	7.2	-	3.00	83
0.50	3	0.00	9.6	-	4.01	84
1.00	3	0.00	9.6	-	3.78	79

However in the pilot plant, or commercially, larger volumes are used and it should be possible to control the larger amount of air more accurately. Also the pilot plant and commercial plant will of necessity be equipped with good recording devices with automatic control. Some of the variability in the fermentation should thus be eliminated in the good commercial practice.

In our laboratory tests no continued successful fermentations were obtained until an easily controlled unlimited source of air was made available. Previous tests were limited by the amount of air that could be supplied to the samples, and the variations in results due to aeration were very significant. The success of these early experiments was limited entirely by our ability to supply adequate aeration.

A good source of air was made available by the use of a unit consisting of an electric motor, a pump, and automatic cut-out and a pressure tank. Air was led out through a constant pressure valve of the type used with high pressure tanks, and a very even control of air pressure was obtained. The air was passed through a sterilized cotton filter, bubbled through water and supplied to the samples as desired. Air was sparged into the samples through capillary tubing mainly, although cotton bags, frittered glass and alundum aerators were all tried. Large test tubes (40 mm. x 295 mm.) were mainly used, with 50 cc. to 150 cc. samples, although in some tests very large tubes (65 mm. x 490 mm.) were used to ferment 1 litre batches. In most cases the bottom was cut out of these extra large tubes and air supplied from the outside through a fine cotton bag covering the rubber stopper.

Rate of aeration was measured by collecting the exhaust gases in a measuring cylinder by the displacement of water, using a stop watch.

It was found that as the fermentation proceeded the medium became more viscous and a greater air pressure was required to maintain the desired aeration rate. No other method of measuring rate of flow was tried that was sensitive enough to give sufficiently accurate results. It is felt that to obtain the necessary aeration control an accurate, automatic system of measuring rate of flow is required.

A great number of experiments have been run considering the amount, effectiveness and results of aeration on the fermentation. It was found that the rate of aeration controlled to a considerable extent the time of fermentation. Aeration also exerts a control on the amount of acetylmethylcarbinol in the final fermentation liquor, expecially where the aeration rate is high in the latter stages of the fermentation.

Table 17 shows the comparative effect of fermentation with cotton bags. These bags were tightly fastened to the lower end of the capillary tubes and the air was broken up into a fine stream of bubbles. Difficulties arising from contamination of the medium were more frequently encountered where these 'aeration' bags were used. Aeration was moderate in all cases and although the glycol yields were low the duplicates did not show great variation. The results show that although aeration with the cotton bags caused a better utilization of the sugar the yields of glycol were lower. It is realized that the air is more efficiently used when broken up into smaller bubbles and quite possibly a lower rate of aeration would have given better results. In the laboratory it is much more convenient to clean and keep sterile capillary tubes and these have been used extensively.

The use of alundum balls and frittered glass aerators gave an excellent distribution of the air into fine bubbles. However in both

TABLE 17

EFFECT OF USING COTTON BAGS AS AERATORS ON DEXTROSE AND ACID-HYDROLYZED MEDIA

Aerator	Medium	# Samples	% Sugar		% Glycol	% Carbinol	% Etoh	'A'	'B'	'C'
			Final	consumed						
Capillary	Dextrose	6	1.23	6.4	2.49	.18	-	78	83	-
Bag	"	6	0.28	7.3	2.07	.34	-	57	66	-
Capillary	Acid Hydrolyzed Wheat	6	1.00	4.1	1.61	.26	.41	78	91	111
Bag		6	0.84	4.3	1.56	.27	.37	73	85	102

cases normal fermentations were not obtained probably due to inhibition of the culture induced by these aerators.

The amount of air used for aeration is very important and a great range of optimum rates has been suggested. However the use of various types of apparatus will vary the amount of air required. Table 18 gives results of aerating at different rates over a period of time. Note the decided change in activity of the culture. Comparing these to the results reported by A.L. Shewfelt (36) also shows the tendency of the culture to vary in rate of fermentation. Heavy aeration gives a fast rate of fermentation, where the culture in active, but a low glycol yield. Under our conditions an aeration rate in the range from 250 to 500 cc. per litre per minute is optimum. This will give a fermentation time from 48 to 60 hours with good yields. A faster time

TABLE 18

EFFECT OF RATE OF AERATION ON THE TIME OF FERMENTATION AND THE YIELD OF GLYCOL FROM ACID-HYDROLYZED AND DEXTROSE MEDIA

Date	Aeration Rate cc./litre/min.	Medium	Time	# Samples	% Sugar		% Glycol	% Carbinol	% Etoh	'A'	'B'	'C'
					Final	Consumed						
Jan./43	500	Acid Hydrolyzed Starch	96	4	.41	7.1	2.82	0.03	-	79	80	-
Feb./43	1000		89	8	1.30	4.0	1.81	0.26	-	91	104	-
July/43	1000	Dextrose	46	6	.31	9.4	3.00	0.53	.60	64	75	88
Aug./43	2000	"	65	10	.59	9.2	2.95	0.06	.54	64	65	77
Dec./43	250	"	66	2	0.00	8.4	2.94	-	-	70	-	-
	500	"	66	3	0.00	8.4	2.94	-	-	70	-	-
Feb./44	500	"	40	6	.98	8.6	3.07	0.00	-	72	72	-
	500	"	49	6	0.00	9.6	3.86	0.00	-	80	80	-

appears to reduce the yields while a longer time of fermentation gives no advantage. In a number of other tables also the effects of the rate of aeration on the yields are noted.

In order to determine the period during a fermentation in which the aeration was most effective a number of experiments were run. Some of the results are reported in Table 19. Dextrose medium was used in all cases and nutrients supplied. It is noted that to complete the fermentation in 46 hours heavy aeration is needed. Any slower rate of aeration either at the beginning or end of the fermentation increased the final sugar and lowered the yield. Slower rates of aeration in the latter stages of the fermentation did not appear to increase the glycol yield. The percent alcohol did not vary consistently and showed little change regardless of aeration treatment. The second part of this table demonstrates the adverse effect of a high aeration rate in a longer fermentation. Undoubtedly the lower glycol yields at the higher aeration rates can be mainly accounted for in higher acetylmethylcarbinol yields. Quite possibly the small amounts of remaining sugar at the higher rates would not be shown if the correction for carbinol was applied. This experiment is a good example of the effect of a high rate of aeration on a fermentation which is allowed to proceed past completion.

Considerable difficulty was encountered using high rates of aeration with foaming tendencies of the medium. The condition of the culture had some effect on this tendency. However malt-hydrolyzed products show a great tendency to foam even when the medium is filtered before use. The medium suggested by Seagram's Laboratories (32) also induces to some extent this tendency.

To counteract this effect anti-foam agents were added. Amyl alcohol

TABLE 19

EFFECT OF VARIED AERATION ON THE FERMENTATION OF DEXTROSE MEDIUM

A.

		#	% Sugar			%				
Aeration	Time (hours)	Samples	Final	Consumed	% Glycol	Carbinol	% Etoh	'A'	'B'	'C'
* Heavy.	46	9	0.55	10.0	2.82	0.43	0.42	56	65	73
Heavy, moderate last 16 hours.	46	6	1.43	9.2	2.56	0.14	0.38	62	63	75
Heavy, none last 16 hours.	46	6	2.44	8.2	1.84	0.00	0.40	45	45	55
Moderate	46	3	2.88	7.7	2.13	0.00	0.44	55	55	67
Moderate for 24 hours, then heavy.	46	6	1.25	9.3	2.86	0.05	0.41	62	63	71
Moderate for 22 hours, heavy for 7 hours, moderate for 16 hours.	46	3	3.58	6.8	1.84	0.10	0.35	54	57	67
Heavy.	40	3	1.62	9.0	2.53	0.06	0.28	56	58	64
Heavy, moderate last 16 hours.	40	3	3.39	7.2	1.87	0.08	0.47	52	54	67

* Heavy aeration is approximately 500 cc./litre/min.
Moderate aeration is approximately 250 cc./litre/min.

B. Time of fermentation = 80 hours.

	#	% Sugar		%	
Aeration	Samples	Final	Consumed	Glycol	'A'
100 cc./litre /min.	6	0.00	10.1	3.44	68
250 cc./litre/min.	6	0.44	9.7	2.72	56
500 cc./litre/min.	6	0.26	9.8	2.77	57

TABLE 20

EFFECT OF ADDED OLEIC ACID ON THE FERMENTATION OF DEXTROSE MEDIUM

Oleic Acid	Aeration	# Samples	% Sugar Final	% Sugar Consumed	% Glycol	% Carbinol	'A'	'B'
A. Time of Fermentation = 68 hours.								
0	Moderate	2	0.99	9.12	2.91	0.00	64	-
5 drops	"	2	2.22	7.78	2.13	0.00	55	-
B. Time of Fermentation = 68 hours								
0	250cc./litre/	3	0.12	9.88	2.82	0.00	57	-
5 drops	" min.	3	3.58	6.42	1.91	0.00	60	-
0	500cc./litre/	3	0.51	9.49	3.29	0.78	69	96
5 drops	" min.	3	0.92	9.08	2.94	0.00	65	-
0	1000cc./litre/	2	0.71	9.29	3.00	1.22	65	91
5 drops	" min.	2	0.14	9.86	3.16	0.39	64	72

was tried, but although effective, the liquid is too volatile and the effect lasts only a short time. It is thought that amyl alcohol reduces the glycol yield. Commercial lard oil was very useful but inhibited the fermentation to a marked extent. The use of oleic acid gave the best results. Except in very few cases the addition of a few drops controlled the foaming sufficiently so that a normal fermentation was obtained. It was thought that the addition of oleic acid to some extent inhibited the fermentation To test this a series of experiments were carried out and representative results are reported in Table 20.

At a lower aeration rate the utilization of sugar is definitely interfered with and the actual glycol yields are lower where oleic acid is

supplied. However at the higher rates of aeration this inhibitory effect is not present. Sugar utilization is good and the yields of glycol are comparable to those of the checks. Note the lower production of acetylmethylcarbinol where oleic acid is added. The combined yield of glycol and carbinol definitely favors the check samples and shows that even at these higher rates of aeration the oleic acid inhibits the fermentation.

It is undoubtedly better to make use, if possible, of a culture and medium which do not favor the production of this undesirable foaming characteristic.

To supply sufficient sterilized air for a large fermentation would be an expensive process and difficult to maintain. Considering this fact a series of 10 tubes was aerated in the following manner. Air was introduced into the first tube at a constant rate. The exhaust air from the first tube was forced through the second tube through rubber tubing. This was repeated through the ten tubes. Leaks were eliminated as far as possible and all the tubes had the same air going through each one plus any carbon dioxide or hydrogen that was produced by the fermentation. N.R.R.L. dextrose medium was used in each case. The results are reported in Table 21.

These results are somewhat variable but there is no significant variation. Thus we can say that the air can be recycled through the fermentation medium at least nine more times without any loss of effectiveness. In our laboratory it is not possible to collect the exhaust air plus carbon dioxide plus hydrogen and recycle it but in commercial practice this should be possible and, if effective this would eliminate to some extent the sterilization of large amounts of air.

TABLE 21

EFFECT OF RE-CYCLED AIR ON THE FERMENTATION OF DEXTROSE MEDIUM

	% Sugar								
No.	Final	Con-sumed	pH	% Glycol	% Carbinol	% Etoh	'A'	'B'	'C'
1	.54	9.3	-	3.67	0.5	-	79	90	-
2	.46	9.3	6.1	3.64	0.5	.43	78	89	98
3	.33	9.5	-	3.68	0.5	.40	77	88	96
4	.36	9.4	5.6	3.71	0.5	-	79	90	-
5	.96	8.8	-	3.65	0.5	.55	83	94	107
6	.31	9.5	5.7	3.84	0.5	-	81	91	-
7	T.	9.8	-	3.77	0.5	-	77	88	-
8	.28	9.5	5.7	3.68	0.5	-	77	88	-
9	1.01	8.8	-	3.32	0.5	-	75	87	-
10	.55	9.2	6.8	3.59	0.5	-	78	89	-

This experiment led to the reconsideration of the suggestion that the air performed only a physical action in the fermentation. A tank of liquid carbon dioxide was obtained from the Canadian Liquid Carbonic Corporation in Edmonton. After preliminary tests an experiment was carried out using various mixtures of CO_2 and air. A sample of the mixture was analyzed in each case and the amount of CO_2 determined accurately. N.R.R.L. dextrose medium was inoculated and the aeration rate controlled carefully. Samples of the fermentation liquors were taken at regular intervals and analyzed. The results are reported in Table 22. In no case was there ever any acetylmethylcarbinol present

TABLE 22

EFFECT OF AERATION WITH CO_2 ON THE FERMENTATION OF DEXTROSE MEDIUM

% CO_2	Aeration Rate cc./litre /min.	# Samples	Time (hours)	% Sugar		% Glycol	% Etoh	'A'	'C'
				Final	Consumed				
.4	250	4	44	1.84	8.0	2.06	-	52	-
	250	4	68	0.00	9.8	2.81	-	57	-
	500	4	44	1.00	8.8	2.81	-	64	-
	500	4	68	0.00	9.8	2.98	-	61	-
25.1	250	4	44	4.03	4.2	1.22	-	58	-
	250	4	68	2.28	5.9	1.81	-	61	-
	250	4	92	0.90	7.3	2.28	0.88	63	87
	500	4	44	2.66	5.5	1.72	-	62	-
	500	4	68	0.60	7.6	2.37	-	63	-
	500	4	92	0.07	8.1	2.40	0.86	59	81
48.1	250	8	44	3.08	5.7	1.87	-	66	-
	250	8	68	2.03	6.8	1.82	-	54	-
	500	8	44	2.02	6.8	2.34	-	69	-
	500	8	68	1.34	7.5	2.19	-	58	-
69.2	250	4	44	0.00	8.2	2.18	-	53	-
	250	4	68	0.00	8.2	2.08	-	51	-
	500	4	44	0.00	8.2	2.46	-	60	-
	500	4	68	0.00	8.2	2.41	-	59	-
100.0	250	4	44	3.80	5.8	2.16	-	74	-
	250	4	68	1.13	8.5	3.04	-	72	-
	500	4	44	4.86	4.7	1.98	-	81	-
	500	4	68	1.87	7.7	3.11	-	81	-
	1000	4	67	2.80	6.8	1.97	-	58	-
	1000	4	108	1.01	8.6	1.97	1.29	47	76

in the fermentation liquor.

Aeration rates as high as 1000 cc. per litre per minute failed to produce any carbinol where the percent CO_2 was high. At 250 cc. per litre per minute aeration with only 25% CO_2 showed no carbinol on analysis. These tests were run consecutively to try to offset any variations in the activity of the culture. However it is apparent that the rate of fermentation is more affected by other factors than by the percent CO_2 used for aeration; possibly by a change in the activity of the culture. Considering all the results no consistent trend in yields is noted with increasing percent CO_2. However at the higher percent CO_2, in each case, the longer time of fermentation gave a rather lower yield of glycol, instead of a higher yield as in the case where air containing 25% CO_2 was used, even though there is more sugar utilized. This fermentation apparently does not favor the formation of glycol but as the results show the percent alcohol formed is considerably in excess of the amount usually present. The fact that the fermentation will take place with CO_2 replacing air in the aeration system is very significant and supports the theory that the effect of the air is a physical phenomenon. Further experiments to find the effect of using CO_2 and a culture giving high yields in the check will probably show the extent to which CO_2 can be used without detrimental effects. If this factor were known the practical extent of recyclization of air through the fermentation medium could be decided.

As it was very difficult to obtain an accurate control of aeration with air other suggested methods were tried.

Mixing with a mechanical stirrer did not give satisfactory results. No apparatus was developed that could be safely left overnight and though

this is feasible, practical considerations did not appear to warrant this development. Other methods of agitating the fermentation medium were tried but none with success, mostly due to difficulties in construction of a suitable apparatus. The most successful of the these involved the use of a revolving drum, but even here yields were low and uncertain.

Shaking the fermentation medium in a 'to and fro' shaker gave variable results. Small variations in the current caused the motor to fluctuate greatly in speed and although it was possible to vary the speed of the motor this required constant supervision and was thus not practical. Another difficulty arose due to wetting of the plugs from splashing. This effect introduced difficulties that could not be readily overcome under our conditions. The method did not give more consistent results and required more supervision than the use of air for aeration. The apparatus was used extensively in the preparation of inoculum solutions where aeration was desired. Some fermentation results are given in Table 23. The effect of shaking the fermentation solution was compared with ordinary moderate aeration on two media. The low yields from the sugar medium are not easily explained. Undoubtedly the initial sugar reported on the acid-hydrolyzed wheat medium is too low. Some difficulties were found in obtaining the correct percent reducing substances in this medium, possibly because the organism is able to ferment some of the higher molecular weight constituents which are incompletely broken down to dextrose. The results from both methods of aeration are very variable. This is an example of one of the better fermentations produced by shaking the medium. Mechanical difficulties limited the usefulness of the method and most of the experiments led to unsatisfactory fermentations.

[illegible] overcome under other conditions. [illegible]

[illegible] The apparatus was used [illegible]

[illegible] The effect of [illegible]

[illegible] was corrected with ordinary [illegible]

[illegible] de from the upper portion are not nearly as good. [illegible]

[illegible] so very windy. [illegible]

TABLE 23

EFFECT OF SHAKING ACID-HYDROLYZED AND DEXTROSE MEDIUM ON THE GLYCOL AND CARBINOL YIELDS

Time of fermentation = 69 hours.

Aeration	Medium	% Sugar Final	% Sugar Consumed	% Glycol	% Carbinol	'A'	'B'
Shaken	Acid)	1.4	5.1	1.82	.40	71	87
"	Hydrolyzed(	1.2	5.3	2.38	.40	90	105
"	Ground)	1.0	5.5	2.21	.40	80	91
Aerated	Wheat (	0.5	6.0	2.53	.25	84	93
"	"	0.8	5.7	2.94	.40	103	117
"	"	0.9	5.6	2.76	.40	98	113
Shaken	Dextrose	1.7	8.7	2.16	.25	50	55
Aerated	"	1.6	8.8	0.43	.00	10	10

Aeration has introduced much variation into our laboratory experimental results and if aeration were unnecessary considerable benefits would result both in laboratory and commercial practice. Therefore Aerobacter aerogenes #199 has been tested from time to time in non-aerated fermentation. (See Table 13 and Table 15.) Table 24 shows the results of some of these check tests and demonstrates the variation that is encountered in the activity of the culture under these conditions over a period of time. In nearly all tests the yields of glycol are lower than those obtained where aeration is used. Note the exceptionally high alcohol yields reported in the last two

TABLE 24

VARIATIONS IN GLYCOL AND ALCOHOL YIELDS IN NON-AERATED DEXTROSE MEDIUM

Date	Time (hours)	% Sugar Final	% Sugar Consumed	pH	% Glycol	% Etoh	'A'	'C'
Nov./42	120	0.8	8.3	5.5	1.97	-	47	-
Jan./43	120	5.1	3.1	-	0.40	-	26	-
July/43	72	0.5	10.5	-	3.77	-	72	-
Nov./43	92	0.5	9.3	-	1.84	1.36	40	69
	164	0.0	9.8	-	2.55	1.30	52	79
Jan./44	96	0.0	5.6	-	1.44	1.10	51	91

tests. The low yields and long fermentation time required where no aeration is supplied make it essential to aerate the samples in order to obtain a normal fermentation.

9. Glycol - Alcohol Ratio

It has been observed over a period of time that there are definite variations both in the activity of the culture and in the relative yields of glycol and alcohol produced. These variations have been discussed previously. In addition to these however it is found that the ratio of glycol to alcohol varies over a period of time. The results found in Table 25 represent this change. Although this appears to be a rather gradual tendency there are marked variations from the general level in certain experiments. These latter variations are very hard to account for unless it is assumed that the culture produces markedly

TABLE 25

THE RATIO OF GLYCOL TO ALCOHOL AS FOUND IN VARIOUS FERMENTATIONS USING DEXTROSE MEDIUM

Date	# Samples	% Glycol	% Alcohol	Ratio
Apr./43	4	2.68	0.82	3.2 : 1
Apr./43	10	1.98	0.39	5.1 : 1
May /43	12	1.60	0.55	2.9 : 1
July/43	10	2.63	0.49	5.4 : 1
Aug./43	10	3.35	0.54	6.2 : 1
Sep./43	6	3.69	0.58	6.4 : 1
Nov./43	8	2.35	0.83	2.8 : 1
Nov./43	4	3.12	0.64	4.9 : 1
Jan./44	5	2.01	0.68	3.0 : 1

different strains from time to time. The general decline of the ratio from August and September, 1943 until January, 1944, is very hard to explain and no controls have been devised that have permanently increased the ratio. By a random selection of colonies from the plated culture, it is possible to obtain certain strains that give higher glycol yields. However this method is only useful within moderate limits. The best method of control probably is to preserve the culture either by infrequent transfer or by lyophilization when the ratio is high.

TABLE 26

THE COMPARATIVE PRODUCTION OF ALCOHOL AND GLYCOL ON DEXTROSE MEDIUM AT VARIED AERATION RATES

	% Sugar								
Time (hours)	Actual	Consumed	pH	% Glycol	% Carbinol	% Etoh	'A'	'B'	'C'
A. Aeration Rate = 1000 cc./litre/min. (Results are given as an average of six tests.)									
1	9.66	0.0	6.0	0.16	.00	.07	-	-	-
16	7.69	2.0	5.7	0.81	.00	.21	81	81	101
24	4.47	4.9	5.3	1.25	.00	.23	51	51	60
46	3.48	6.2	5.1	2.51	.10	.29	81	84	94
56	3.18	6.5	5.1	2.58	.05	.27	79	81	89
64	1.27	8.4	5.0	2.68	.10	.39	64	66	75
B. Aeration Rate = 1500 cc./litre/min. (Results are given as an average of two tests.)									
1	9.33	0.0	5.9	0.09	.00	.05	-	-	-
16	8.46	0.9	5.4	0.40	.00	.19	89	89	131
24	5.57	3.8	5.2	1.20	.00	.18	63	63	73
46	2.53	6.8	5.2	2.16	.02	.28	63	64	72
56	1.11	8.2	5.1	3.04	.01	.26	74	74	81
64	0.67	8.6	5.1	3.13	.03	.36	73	74	82

10. Yield Curves

Some experiments were run in very large test tubes fermenting about 1000 cc. in each sample. Tests were run at various rates of aeration and much care was used to standardize conditions. Because of the larger volume of air required a closer control of aeration rate was possible. Samples were removed at various intervals and analyzed. Table 26 gives some of the results obtained. See also the accompanying graph. N.R.R.L. dextrose medium was used in all cases and salts, urea

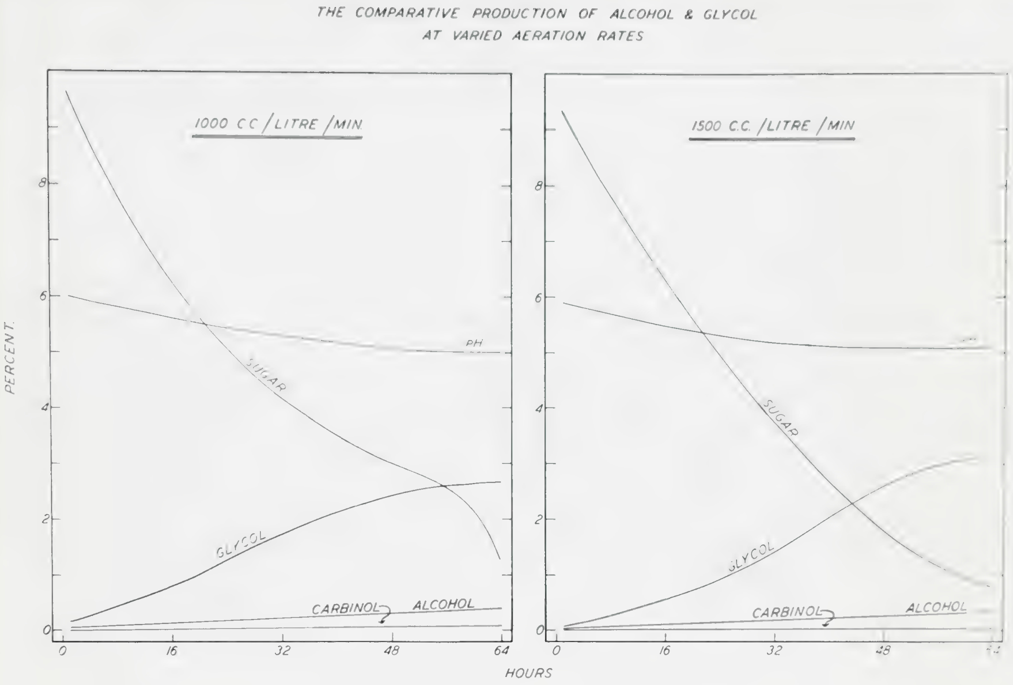

THE COMPARATIVE PRODUCTION OF ALCOHOL & GLYCOL
AT VARIED AERATION RATES
1000 CC /LITRE /MIN.
1500 C.C. /LITRE /MIN
PERCENT.
0
2
4
6
8
HOURS
0
16
32
48
64
PH
SUGAR
GLYCOL
CARBINOL
ALCOHOL

PLATE 4.

and $CaCO_3$ supplied. Foaming was a problem in some of the tests and necessitated the addition of oleic acid which may account for the rather low yields. Heavy aeration did not produce the quick fermentation and high yields of carbinol that were obtained under similar conditions, when 100 cc. samples were tested. The sugar was gradually utilized, until it fell to a low level although the fermentations were not quite complete at 64 hours. The pH values dropped rapidly in the first 24 hours then changed but little more. Glycol and alcohol are both produced gradually as the sugar is utilized and approach the maximum amount obtainable towards the end of the fermentation. Acetylmethylcarbinol was produced only in very small amounts towards the end of the fermentation. The graph shows these effects very well and demonstrates the important fact that as the sugar is gradually utilized it is converted to glycol and alcohol in about the same ratio throughout the fermentation. Even in these large samples variations between replicate tests were quite marked although it is felt that a better control is possible than when using smaller samples.

Isolation and Study of New Organisms

1. Isolation Technique

After making a preliminary survey of the literature on isolation technique and obtaining suggestions from other laboratories (4, 38) the following method of isolation was adopted.

The contaminated materials (soils, fresh or rotting vegetables, etc.) are placed in 10 cc. of Peptone-Lactose Bile Broth (19) in a test tube and stored in a 30°C. incubator for 24 to 48 hours. It was found that dextrose may be substituted for lactose with equal success. Good gas production in this medium is an indication of an active culture. 1 cc. transfers are made from the original test tube to another tube containing the same medium and incubated for 24 hours. The mixed culture is then plated on either Difco Eosin Methylene-Blue Agar or on Difco MacConkey's Neutral Red Agar. By experience it was possible to select the correct organisms very easily and on Difco Methylene-Blue Agar the standard type of *Aerobacter* colonies if well separated were quite large (4 - 6 mm.); neighboring colonies ran together quickly; colonies are considerably raised and markedly convex; the centers are deep brown and usually no metallic sheen is observed. On MacConkey's Neutral Red Agar two main types of *Aerobacter* colonies were observed:

1. Small red colonies producing no color change in the medium.

2. Lighter coloured colonies (yellow or pink) having a definite thick centre and pale outer ring accompanied by an alkaline reaction in the medium.

After selection, each colony was transferred to N.R.R.L. inoculum medium. It was found that very few types of organisms other than *Aerobacter* were present on the plates. The original source does not

favor growth of *Escherichia* and other contaminants were inhibited by the various media. A variety of colony types was observed on both media but the most desirable organisms appeared to have the standard characteristic colony type (4, 8).

After growth had taken place in the N.R.R.L. inoculum the selection was tested in Kosen-Citrate medium (8). Growth at 24 hours as indicated by a turbid solution was considered a positive test. However after preliminary tests, it was felt that this step was unnecessary in that the results were somewhat variable mainly because the test is hard to determine when only slightly positive.

The Voges-Proskauer test became the critical test. After much investigation of different suggested methods the modification suggested by Dorner and Hellinger (9) was used and a positive test was taken to indicate the definite presence of an *Aerobacter* culture.

In all, 341 cultures classified as *Aerobacter* types, were isolated and to these, yield tests were applied.

2. Selection of Suitable Culture

As it became apparent that the necessity for aeration was one of the limiting factors in the *Aerobacter* fermentation it was resolved to try to isolate a culture which would give a reasonably active fermentation with a good yield of 2,3 butylene glycol in a non-aerated medium. The use of a non-aerated medium considerably limited the amount of routine labor required. By experiment it was found that isolates giving the best results in non-aerated medium gave the highest yields in aerated medium.

All isolates were tested at least in duplicate, using test tubes samples containing 10 cc. of N.R.R.L. dextrose fermentation medium (26). These tubes were analyzed on the fifth and if necessary the ninth day. A quick test to give the general level of sugar remaining was utilized.

A 1 cc. sample was added to 5 cc. of Benedict's solution, shaken, and heated in a boiling water bath for two minutes. The colour of the solution indicated the general sugar level.

If the sugar was low 2,3 butylene glycol, and in many cases ethyl alcohol, were determined. From this first selection 75 cultures were picked as having good possibilities. All gave fair yields in a 5 day fermentation. The criterion was set at an average glycol level of 2% in these first tests.

Many of these selections were tested with aeration and comparative yields were obtained. Further selection was necessary and a series of tests was carried out on these 75 isolates. After a critical examination of all the results obtained 20 cultures were selected for further study. These cultures have all averaged at least 2.50% glycol without aeration, which is between 50% and 60% of theoretical production based on the initial amount of sugar supplied. In most cases the alcohol yields are high and the glycol alcohol ratio varies from 5 : 1 to 1.5 : 1 in the non-aerated medium. The time of fermentation under optimum conditions was reduced to 3 days in many tests.

3. Glycol and Alcohol Yields

Table 27 gives some of the yields obtained with the better selections both on aerated and non-aerated medium. Where no aeration was used the fermentation took from 3 to 7 days but where aeration was

TABLE 27

FERMENTATION RESULTS OBTAINED WITH SELECTED ISOLATES

(All tests run on N.R.R.L. Dextrose Medium)

A. No aeration

No.	# Tests	% Glycol High	Low	Average	% Etoh	'A'	'C'
196	6	4.21	2.08	2.89	1.32	61	90
197	6	3.79	2.44	2.80	1.24	60	65
198	6	3.92	2.49	2.99	1.30	64	91
249	8	3.51	2.33	2.62	0.51	56	67
250	9	3.49	2.10	2.58	0.54	55	67
256	7	3.69	2.21	2.63	0.46	56	66
258	9	3.14	1.73	2.61	0.80	56	73
260	9	3.44	1.93	2.69	0.47	57	67
265	5	3.09	2.39	2.68	0.54	57	69
267	7	3.69	1.14	2.42	-	52	-

B. Moderate Aeration

No.	Time (days)	% Sugar Final	Consumed	% Glycol	% Carbinol	% Etoh	'A'	'B'	'C'
250	3	0.5	9.1	2.99	0.00	0.39	66	66	74
258	4	0.5	9.1	3.21	0.02	0.53	71	77	82
260	4	0.5	9.1	3.44	0.02	0.39	76	76	85
265	4	1.0	8.6	3.04	0.50	0.47	71	82	93

used the fermentation was complete in 3 to 4 days. Aeration appears to inhibit alcohol production and aid in glycol production, particularly in some cases. There is no great variation in results between cultures and it was noticed that in any one test the general level of glycol yields varied little among the selected isolates. There was a definite variation in yields between tests of one isolate. The yields with aeration compare somewhat favorably to those obtained using selection #199, although the glycol yields are considerably lower than the better returns from this selected culture. It is not felt that any one of these isolated cultures possesses any significant or distinct advantage over Aerobacter aerogenes #199.

Three of the selections (198, 249, and 262) were tested in non-aerated medium using various amounts of salts and urea. (See Table 13B and Table 15 for #199 results.) Various amounts of salts did not produce any definite trend. However, increasing amounts of urea, as with #199, increased the amount of glycol produced by all three selections.

Some tests were run on large volumes (1000 cc.) with no aeration in order to compare yields with those obtained with small samples. It was found that the comparative glycol yields were somewhat lower but did not vary markedly in these larger samples.

DISCUSSION

Aerobacter aerogenes #199 is a very active culture which will under optimum conditions produce a relatively quick fermentation giving a good yield of 2,3 butylene glycol.

To find these optimum conditions so that a rapid fermentation with high yields may be attained, all the major factors which affect the fermentation have been considered in detail. Various media have been tested under many conditions and the results reported.

No one factor acts independently of the others and thus, to secure the desired result, it is necessary to have all conditions at an optimum. Due to this fact, the study of this fermentation becomes more involved, in that, unless all other factors except the one under consideration are optimum, the correct effect is not attained. Because these factors cannot be kept at optimum very variable results are obtained and it is necessary to run a number of samples and tests in one experiment and average the results. Acid and malt-hydrolyzed media have been extensively prepared from ground wheat, flour, granular flour and starch and used in the fermentation. Suitable laboratory methods of preparation were studied using both acid and malt, Both brewer's and distiller's malt were used although it was found that distiller's malt gave the better results. Malt-hydrolyzed medium was more easily prepared and gave a better fermentation than acid-hydrolyzed medium. Granular flour was found to possess some definitely favorable characteristics that made it very useful. In fermentation experiments acid-

hydrolyzed and malt-hydrolyzed media gave more variable results than dextrose medium and for that reason the latter medium has been used extensively in studying the various factors.

The organism has been found to vary seriously over a period of time for no apparent reason. This has been a variation involving both the rate of fermentation and the yields produced. The rate of fermentation was found to increase under similar aeration rates. Some part of this decrease in time of fermentation is due to better technique in handling the culture and inoculum, and to the use of a more nearly optimum medium. The variation in yields has not only been a lowering of the percent conversion of the sugar utilized to glycol but has also been an increase in the percent converted to alcohol thus tending to lower the glycol to alcohol ratio. This is a very serious problem and the conditions which influence the change are not understood. Two methods of control have been applied.

First by a random selection of the plated culture, it is possible to a limited extent to obtain a strain which will give somewhat better glycol yields.

Secondly it is hoped by using a modified 'lyophile' method to preserve the culture for use, at any time, when it is in a very active condition and produces high glycol yields.

The pH must be within the range 5.0 - 6.5, but if possible should be between 5.2 - 6.0. A pH less than 5.0 inhibits the culture, and sugar utilization and yields are very low. After carrying out the fermentation in the range of temperature from 30°C. to 36°C. it became apparent that other factors affected the results more definitely than did temperature. A temperature of 30°C. is suggested, as good results

can be obtained and it is easier to maintain this lower temperature.

Time of fermentation and rate of aeration are very closely related. A high rate of aeration gives a quick fermentation but the yields are low. A moderate rate of aeration will undoubtedly give more satisfactory returns. The effect of aeration is apparently not due to a chemical reaction but to a physical phenomenon. Air may be recycled with no loss of effect. It was found by experiment that CO_2 either pure or in mixtures with air inhibits the fermentation rate only slightly, but does lower to some extent the glycol yields. This suggests a practical application in that a considerable amount of the air may be recycled giving good results and thus a saving would be made in sterilization of large quantities of air.

It was found very difficult to control the rate of aeration within narrow enough limits so that any variability in the fermentation due to other conditions under question would not be masked by the relatively large amount of variability introduced by irregular aeration. Numerous methods of control were tried and it is felt that there is a definite need for some reliable automatic system of controlling air flow if accurate results are to be attained. Shaking the fermentation medium and other forms of agitation gave no better results than did those where aeration was used.

The nutrition of the culture appears to be very simple. Small amounts of $MgSO_4.7H_2O$ and KH_2PO_4 are very necessary especially where dextrose medium is used. The best source of nitrogen is urea as the culture is able to utilize this source easily without the formation of undesirable by-products. There is some evidence that increasing amounts of urea will increase the rate of fermentation.

By using suitable technique and media it was possible to quickly isolate large numbers of Aerobacter type organisms. The better cultures were selected and tested under a variety of conditions. It was realized that it would be very useful to isolate an organism that would give an active fermentation and good yields without aeration. With this in mind the better isolates were tested and although it was possible to obtain a complete fermentation in from 3 to 7 days the yields of glycol were low and the alcohol yields high. Using these same organisms with aeration increased the yield of glycol significantly and also lowered the fermentation time. No isolate appeared to give better results than Aerobacter aerogenes #199.

It is not possible to evaluate laboratory results in definite terms of commercial practice. This may only be done with any degree of accuracy after the findings in the laboratory are tested in the pilot plant. Thus it is impossible to state, except in general terms, whether under commercial conditions differences in aeration, pH etc. will have the same effects on the fermentation.

It is hoped that the laboratory investigations carried on here and elsewhere will be of definite value in suggesting optimum fermentation conditions that will enable pilot and commercial plants to obtain good results.

SUMMARY

Aerobacter aerogenes #199 is a very active culture giving a rapid fermentation with a relatively good yield of 2,3 butylene glycol and a low yield of alcohol under optimum conditions.

Considerable variation has been encountered both in the activity of the culture and in the yields produced.

By selection of colonies and by 'lyophilization' of desirable cultures, attempts have been made to standardize culture activity and consistently obtain good yields.

Other factors have been tested in order to determine their effect on the fermentation.

In handling the inoculum, transferring the culture from the slant two or three times in inoculum medium before use and using 5 - 10% of a 16 - 24 hour inoculum has given good results.

A fermentation temperature of 30°C. has been found to be satisfactory.

Aeration is essential for a desirable fermentation. Large quantities of sterile air are necessary. Heavy aeration gave a fast fermentation with low yields. Moderate aeration gave a longer fermentation , but more satisfactory yields. It was found feasible to recycle the air at least 10 times with no loss of effect. Aeration with mixtures of CO_2 and air gave variable results with a slight tendency towards lower glycol and higher alcohol yields. No carbinol was present in the final fermentation liquor when using CO_2 aeration. Some satisfactory fermentations were obtained using 100% CO_2 for aeration. Aeration apparently exerts a physical effect on the fermentation.

Shaking and agitation of the medium did not give better returns than aeration.

Oleic acid controlled foaming tendencies but affected the yields to some extent.

The optimum fermentation pH range has been found to be from 5.2 to 6.0, although the range from 5.0 to 6.5 supported growth of the culture. $CaCO_3$ was sufficient to buffer dextrose medium but adding both $CaCO_3$ and Na_2CO_3 was necessary when using acid or malt-hydrolyzed medium.

The techniques used in preparation of both these media have been discussed.

Added 2,3 butylene glycol inhibited the fermentation.

Small amounts of salts and urea were found necessary for the nutrition of the organism using dextrose media. The effect of salts was less marked where malt-hydrolyzed media were used. Autoclaving the urea had no effect on the yields. Some of the tests indicated that a somewhat larger supply of urea may be advantageous. Malt sprouts were not found useful in the fermentation.

The glycol:alcohol ratio apparently decreased over a period of time. No good method of preventing this decrease has yet been found.

Isolations of *Aerobacter* type organisms were selected from local sources. From 341 isolated, 20 have been selected as being superior, but no successful fermentation without aeration giving good glycol yields has been attained. Aeration of these cultures increased the glycol yield and lowered the alcohol yield. No isolate has given better results than can be obtained with *Aerobacter aerogenes* #199.

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APPENDIX

TABLE OF CONTENTS

Page

Summary of the Combined Method for Glycol and Carbinol Estimation 1

The Determination of Reducing Sugars 2

The Determination of Reducing Sugars in Malt-hydrolyzed Media . . 4

Method for Determining Ethyl Alcohol 4

Note: This appendix contains a brief summary of the most important analytical procedures for determining quantitatively the end products of the fermentation. These methods were obtained through the courtesy of W. G. Corns and were found to be the most satisfactory procedures for each determination. For a complete discussion of these and related procedures refer to the M.Sc. thesis of W. G. Corns (7).

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SUMMARY OF THE COMBINED METHOD FOR GLYCOL AND CARBINOL ESTIMATION

A 3 cc. sample of supernatant fermentation liquor is pipetted into a 100 cc. volumetric flask and made up to approximately 70 cc. with water. 5 cc. of a 20% solution of $CuSO_4$ is added, followed by 5 cc. of a 20% suspension of $Ca(OH)_2$. The mixture is gently shaken and allowed to stand for 20 minutes after which it is made up to volume. An aliquot of about 25 cc. is centrifuged. The remainder contained in the volumetric flask is placed in a boiling water bath for 15 minutes, cooled, and an aliquot centrifuged. Both aliquots are prepared for titration, the first one (no bath treatment) without any delay. 2 cc. of supernatant liquid are pipetted into a 125 x 35 mm. test tube followed by 2 cc. of 1.0 N H_2SO_4, and 5 cc. of .01 M HIO_4. This is allowed to stand at room temperature for approximately 15 minutes. Then 10 cc. of 0.5 M $NaHPO_4$, and 1 cc. of 30% KI and some starch indicator are added.

The mixture is titrated immediately with 0.005 N $Na_2S_2O_3$ to the disappearance of the blue color.

A blank determination gives a titration of about 15.5 cc.

Since 1 cc. of .005 N $Na_2S_2O_3$ is equivalent to 0,225 gm. of glycol the calculation is as follows:

(Blank titration minus sample titration in cc.) x 0.375 = % glycol.
The same factor has been used in carbinol calculation, considering the difference obtained between bath treated and non-bath treated aliquots as carbinol.

The above method was found to be reliable for fermentation liquors containing up to 4% sugar where only small amounts of carbinol are

present, and has been found reliable when at least 2% sugar and approximately 1.00% carbinol are encountered in the same solution. Higher concentrations of sugar can be removed by using greater quantities of $CuSO_4$ and $Ca(OH)_2$. In such cases acid and buffer are adjusted accordingly for a clear end point.

THE DETERMINATION OF REDUCING SUGARS

(From the Northern Regional Research Laboratory Methods)

Principle

The method is a modified Shaffer-Hartmann procedure.

Reagents

(a) N/10 potassium dichromate solution (4.001 gm. in 1 litre)

(b) Starch indicator solution, 0.5%

(c) Alkaline copper solution
$CuSO_4.5H_2O$, 25 gm. per litre
sodium citrate $5.5\ H_2O$, 90 gm. per litre
sodium carbonate (anhydrous) 50 gm. per litre

Dissolve $CuSO_4$ separately, pour slowly into solution of sodium salts with stirring.

(d) Iódide - iodate KI, 60 gm. per litre
- KIO_3 - 5.4 gm. per litre

Add 1 gm. of NaOH per litre to stabilize.

(e) 5 N sulphuric acid solution (278 cc. conc. H_2SO_4 per 2 litres)

(f) Potassium oxalate solution (330 gm. $K_2C_2O_4$ per litre)

(g) N/10 sodium thiosulphate solution (24, 82 gm. $Na_2S_2O_3.5H_2O$per litre)

Add 2 gm. $NaHCO_3$ per litre.

50 ml. of the alkaline copper reagent is pipetted into a 300 ml. Erlenmeyer flask. An aliquot of the solution to be tested containing 25 to 150 mgm. of sugar is added, together with distilled water to bring the total volume to 100 ml. The flask is placed on a hot plate adjusted in such a way as to bring the solution to boiling in 4 minutes. The flask is then boiled for 5 minutes; during boiling small beakers are inverted over the flasks to prevent evaporation. The flasks are cooled in a water bath to at least 40 to 50°C. From a pipette exactly 25 ml. of KI -KIO_3 solution is added, together with 20 ml. of 5 N H_2SO_4 added carefully, and 20 ml. of $K_2C_2O_4$ solution. The solution is well shaken and allowed to stand about half a minute until it is clear. It is then titrated with N/10 $Na_2S_2O_3$ using starch solution as indicator. A blank consisting of 50 ml. of alkaline copper reagent and 50 ml. of distilled water is analyzed at the same time.

Calculation

(Blank - thiosulphate titration) x 6.357 = mgm. of Cu.

From a graph previously constructed the mgm of Cu can be read off as mgm. of sugar directly.

Interfering substances

Acetylmethylcarbinol is capable of reducing copper salts and makes the apparent glucose result too high. The correction factor is applied as follows:

Apparent glucose minus (% acetylmethylcarbinol x 0.87) = true glucose

THE DETERMINATION OF REDUCING SUGARS IN MALT-HYDROLYZED MEDIA

(Reference A.O.A.C. - 5th Edition)

Method

Take a 5 cc. sample of the solution and add 85 cc. H_2O and 10 cc. 2.0 N HCL or H_2SO_4. (This gives a 0.2 N HCL or H_2SO_4 solution). Autoclave the acid mixture in an Erlenmeyer for ½ hour at 15 lbs. pressure. The maltose present will be completely hydrolyzed to dextrose. When cool add 0.1 N NaOH until neutral to litmus.

Determine the reducing sugar by the modified Schaffer-Hartmann procedure. Report the results as percent dextrose.

METHODS FOR DETERMINING ETHYL ALCOHOL

Distillation Method (from N.R.R.L. Methods)

Apparatus

A 500 ml. boiling flask; a Kjeldahl adapter with a 50 ml. separatory funnel connected to a coil condenser 15 inches long; a 200 ml. pressure bottle; a water bath.

Reagents

(a) 0.2 N $K_2Cr_2O_7$ (9.801 gm. per litre)

(b) 0.2 N $NaS_2O_3 \cdot H_2O$ (49.638 per litre)

(c) 10% potassium iodide

(d) 50% H_2SO_4

(e) Starch indicator

The fermented mash is centrifuged and a 25 ml. sample of the supernatant liquor removed and placed in the 500 ml. boiling flask along with 1 ml. of 6N NaOH and 25 ml. of water. The stopper, fitted with Kjeldahl adapter and separatory funnel , is put in and connection made with a vertical coil condenser. The receiver is a 50 ml. volumetric flask. The contents of the flask are distilled at such a rate that about 30 ml. of the liquid comes over in 20 to 25 minutes. At the same time water is run into the distillation flask at such a rate that there is no change in volume during the distillation. The receiving flask is made up accurately to 50 ml. and analyzed directly or diluted if necessary.

One ml. of the alcohol-containing distillate (with not more than 30 mgm. alcohol) is placed with 15 ml. of 0.2 N potassium dichromate and 20 ml. of 50% sulphuric acid in a 200 ml. well stoppered pressure bottle, which is well shaken and placed in a boiling water bath for 20 minutes. It is then cooled and diluted to 150 ml. with water, after which 10 ml. of 10% potassium iodide is added. The free iodine is titrated with 0.2N $Na_2S_2O_3$, using starch as an indicator.

Calculation

.0048 (15-titration (ml. of 0.2N $Na_2S_2O_3$) x 100 = gm. alcohol in 100 ml. of original solution)

46 gm. alcohol = 4 litres N thiosulphate

Interfering Substances

Any substance which yields volatile products on oxidation by dichromate will interfere. In the fermentation cultures acetyl-

methylcarbinol and 2,3 butylene glycol are the only interfering compounds. It has been found by experiment that the distillation method used for the recovery of the alcohol separates out almost all the butylene glycol and a large proportion of the acetylmethylcarbinol.

90 gm. butylene glycol = 6 litres normal thiosulphate

88 gm. acetoin = 4 litres normal thiosulphate

Corrections

In routine tests the correction for butylene glycol is negligible. For each 1% acetylmethylcarbinol is the original fermentation solution 0.014% must be subtracted from the final alcohol results.

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